THE INTERACTION BETWEEN NUTRITION AND INSECT STRESS RESPONSE IN A COTTON MODEL SYSTEM

A Dissertation

by

CARRIE ANN DEANS

Submitted to the Office of Graduate and Professional Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Chair of Committee,	Spencer Behmer
Co-Chair of Committee	Gregory Sword
Committee Members,	Megha Parajulee
	Keerti Rathore
	Kenyon Zhu-Salzman
Head of Department,	David Ragsdale

August 2015

Major Subject: Entomology

ABSTRACT

The ability of organisms to deal with adversity is essential for survival, reproductive success. Nutrition has strong impacts on all physiological processes, including stress responses. Several studies have shown diet macronutrients (protein and carbohydrates) have significant effects on the ability of insect herbivores to deal with toxins; however, despite the economic importance, few studies have focused on how macronutrients may impact a pest's susceptibility to insecticides. Therefore, the general goal of this dissertation was to use cotton as a model system to explore the impact of nutrition on susceptibility to an insecticide stressor.

To do this, I first characterized cotton as a resource. This was achieved by measuring the variability in dietary protein (p) and carbohydrates (c) across plant tissues, genotypes, developmental stages, and growing conditions (Chapter II). Secondly, I determined the macronutrient requirements of two polyphagous pest species, the sucking pest *Lygus hesperus* (Western tarnished plant bug) and the chewing pest *Helicoverpa zea* (cotton bollworm) (Chapters III and IV). Finally, I determined the effect of dietary macronutrients on the susceptibility of *H. zea* to the endotoxin Cry1Ac, produced in transgenic *Bt* cotton (Chapter V).

Our results showed that even in an agricultural monoculture, like cotton, there is high variability in p and c content, across tissues, throughout plant development, and between different growing conditions. Despite this variability, I found that both *L. hesperus* and *H. zea* feed selectively to ingest a specific food p:c ratio, or intake target

(IT). Both species selected for a slightly p-biased IT of 1.2:1 (*L. hesperus*) and 1.6:1 (*H. zea*). Dietary macronutrients also had significant impacts on survival and performance for *H. zea* when Cry1Ac was present. Larval survival and performance were best on diets close to the IT; however, at higher concentrations of Cry1Ac, total macronutrient concentration was the most important dietary factor. The diet that produced the worst performance under control conditions, produced the best performance when Cry was present, identifying an interesting physiological trade-off between. These results show that nutrition can effect pesticide efficacy may be more important than currently acknowledged as an environmental mediator of resistance.

DEDICATION

This dissertation, and all the work contained in it, is dedicated to my family, particularly my parents Ron and Julie Deans and my aunt and uncle Christine LaBlanc and Ed Hinchman. Without their guidance and sacrifice I would have never had the opportunity to pursue a graduate degree. I owe all my current and future successes to their enduring support and encouragement.

ACKNOWLEDGEMENTS

First and foremost, I would like to thank my advisors, Dr. Spencer Behmer and Dr. Gregory Sword, as well as my committee members, Dr. Keyon Zhu-Salzman, Dr. Megha Parajulee, and Dr. Keerti Rathore, for their guidance and support throughout the course of this research. It has been a pleasure working and getting to know all of you. Your mentorship has been integral to the success of these projects, and I will use the wealth of information you have given me through the rest of my career. Thank you.

I must thank all of my friends and colleagues, as well as all my lab members. Thank you : Marion Le Gall, Paul Lenhart, Rebecca Clark, Diana Castillo, Cesar Valencia, Josephine Antwi, Adam Landon, Jerry Huntley, and Tyler Raszick. You all made my time here a lot of fun.

Thanks also goes to the Entomology faculty and staff for making my time at Texas A&M University a great experience, as well as Michael Manson and Keith Maggert, from the Biology Department. I also want to extend my gratitude to C. Everette Sayler, whose endowed scholarship financially supported my research throughout my dissertation.

TABLE OF CONTENTS

Page

LIST OF FIGURES

LIST OF TABLES

CHAPTER I

INTRODUCTION

The ability of organisms to deal with adversity is essential for survival, reproductive success, and thus, fitness. Because of this, traits involved in mitigating different kinds of stressors have evolved under strong selection (Bradshaw and Hardwick, 1989; Bijlsma and Loeschcke, 1997; Badyaev, 2005) and represent some of the most highly conserved pathways (Feder, 1999; Kregel, 2002; An et al., 2003; Kültz, 2005; Lushchak, 2011). Stress responses can be very costly in terms of resources, so often these responses are induced when needed rather than constitutively expressed. This flexibility allows organisms to respond dynamically to their environment.

Nutrition, or the process of acquiring the necessary resources for fueling physiological processes involved in survival, growth, and reproduction, has an overriding influence on all organismal processes, and, as such, can be a strong mediator of stress responses. Much work has been done in determining the nutritional requirements for different species under control conditions in the absence of stress; however, it is becoming increasingly clear that nutritional requirements are often context-dependent, meaning that an optimal diet under control conditions may not be optimal under stressful conditions (Lee et al., 2006; Cotter et al., 2008; Povey et al., 2008; Boggs, 2009; Cotter et al., 2010). The fact that different nutrients, or different concentration and/or ratios of nutrients, are needed to successfully contend with different

kinds of stress provides underlying support for the idea that nutritional plasticity is key to dealing with stress.

For insect herbivores, nutrition and stress are intimately related because it is virtually impossible for an insect herbivore to feed on a plant without ingested some kind of allelochemical. As a result, resource quality is largely determined by the balance of nutrients and defensive chemicals in host plants (Berenbaum, 1995; Hägele and Rowell-Rahier, 1999). Studies have shown that the interaction between nutrient content and allelochemicals have a strong influence on insect community structure and evolution (Ehrlich and Raven, 1964; Berenbaum, 1983; Jermy, 1984).

Insects are adept at sensing concentrations of both nutrients and toxins in their food, and feeding behavior is often driven by decision rules that aim to maximize nutrient intake while minimizing allelochemocal ingestion (Slansky and Wheeler, 1992; Bernays et al., 1994; Bernays and Minkenberg, 1997; Singer et al., 2002; Behmer et al., 2002). The geometric framework, which offers a standardized way to conceptualize and test nutrition-related hypotheses, has been extremely useful for exploring nutrient regulation in animals, but particularly in insects (Simpson and Raubenheimer, 1995; Behmer, 2009; Raubenheimer et al., 2009). Nutrient-sensing in insects predominantly relies on chemosensors that respond to amino acids and sugars (Schoonhoven, 1968; Bernays and Chapman, 1994; Behmer, 2009). Studies have also shown that protein (p) and carbohydrates (c) are required in the largest concentrations and have the most significant impacts on survival and performance (Behmer, 2009; Simpson and Raubenheimer, 2012). Given the close tie between these macronutrients and fitness, it is

perhaps not surprising that virtually all the organisms that have been tested show some degree of regulation for both p and c (Simpson and Raubenheimer, 2012). Allowing individuals to choose between two diets that vary in their balance of p and c (p:c ratio) and then measuring consumption of p and c across diet pairings can identify active regulation by the consumer to achieve a specific intake ratio of these two macronutrients. This self-selected p:c ratio is called an intake target (IT). Intake targets can vary by species, and sometimes populations, but because they are self-selected they indicate the balance of macronutrients that is optimal for maximized fitness (Simpson et al., 2004; Behmer, 2009; Simpson and Raubenheimer, 2012).

Interest in the interaction between nutrition and stress is growing; however, relative little work has been done on making connections between specific nutritional components and specific stressors. There is a wealth of information available on dietary restriction and how diet may increase longevity (Masoro, 1993; Yu, 1996; Mobbs et al., 2007; Mair and Dillin, 2008); however, this phenomenon refers to very specific and rather anomalous occurrence. As a result, our current understanding of dietary restriction has limited relevance to more common stress conditions. The more relevant work that has been done using a geometric framework focuses on nutritional effects on both detoxification and immunity. Simpson and Raubehenheimer (2001) and Behmer et al. (2002) both looked at the effect of diet macronutrients and the grass allelochemical tannic acid on survival and performance in the locust. They found that survival and performance were highest on the diet that most closely matched the IT for that species; however, they did find that negative effects were more intense on the c-biased diets than

the p-biased, indicating that p may play a unique role. Other studies focusing on immunity have found that dietary p is integral for mounting an effective immune response in insects, with infected individuals actively regulating for higher p content in their diet (Lee et al., 2006; Cotter et al., 2008; Povey et al., 2008; Boggs, 2009; Cotter et al., 2010). These studies make a strong case for macronutrients having a primary role in mediating stress responses.

Agricultural systems provide a great opportunity to explore the interplay between macronutrients and stress in insect herbivores for several reasons. First, plant macronutrient content is highly variable, both spatially and temporally, offering insects a broad range of nutritional options to choose from (Elser et al., 2000; McGroddy, Daufresne, and Hedin, 2003). Second, insecticides, which are commonly used in agricultural cropping systems, represent strong stressors. Lastly, agricultural pests have significant economic impacts on crop production throughout the world. With the world population now over 7 billion, food security is a high priority (Godfray et al., 2010). Insect resistance, whether environmentally-mediated or genetic, poses a serious threat to our ability to maintain and/or increase food production. Understanding the nutritional ecology of important pest species, and the factors that impact insecticide efficacy, such as nutrition, has direct relevance to effective pest management.

This dissertation describes several experiments that are focused on understanding the impacts on nutrition on insect stress response in an agricultural system. The second chapter documents the macronutrient variability available to insect herbivores in cotton (*Gossypium* spp.) by measuring p and c content across different tissues within a plant, as

well as across plant gentoypes, plant development, and growing conditions. The third chapter focuses on understanding macronutrient regulation in the polyphagous sucking pest *Lygus hesperus*, a common pest of cotton. The fourth chapter focuses on reassessing macronutrient regulation in the polyphagous pest *Helicoverpa zea*, or the cotton bollworm. The fifth and final chapter uses *H. zea* and transgenic *Bt* cotton as a model system for exploring how diet macronutrient content affects susceptibility to Cry1Ac, an endotoxin from *Bacillus thuringiensis* expressed in transgenic cotton. Taken together, these chapters combine elements of plant physiology, insect nutritional ecology, insect physiology, and toxicology to provide a well-rounded examination of nutrition-stress interactions.

CHAPTER II

SPATIO-TEMPORAL, GENOTYPIC AND ENVIRONMENTAL EFFECTS ON PLANT PROTEIN AND DIGESTIBLE CARBOHYDRATE CONTENT: IMPLICATIONS FOR INSECT HERBIVORES WITH COTTON AS AN EXEMPLAR

Overview

Food protein and digestible carbohydrate content significantly affects insect herbivore fitness, but studies reporting plant protein and digestible carbohydrate content are rare. Instead, nitrogen (N) and carbon (C) are often used as surrogates for plant protein and digestible carbohydrates. This is problematic for two reasons. First, while N loosely correlates with protein content, C shows no relation with digestible carbohydrate content. Second, insect herbivores regulate and utilize protein and digestible carbohydrates, not N and C. The goal of this study was to provide a more relevant context for understanding insect herbivore nutritional ecology by characterizing plant macronutrient content across different plant tissues, varieties (genotypes), and growing environments. We used cotton as a model and measured the soluble protein (P) and digestible carbohydrate (C) content of four plant tissues: (1) young leaves, (2) mature leaves, (3) developing flowers and (4) fruits. We then compared tissue macronutrient variation across plant age for two species and four varieties (genotypes) in two different environments (greenhouse and field). When plant tissues were compared, significant differences were observed in: (1) P and C concentrations, (2) total macronutrient content (P+C concentrations), and (3) P:C ratio. Plant age and environment had significant

effects on these variables, while genotype had a weaker effect. We also observed fine-scale nutrient dynamics in more complex tissues, such as bolls. Foliar tissues had higher total macronutrient content than reproductive tissues, except for developing seeds which on average contained 2x the total macronutrient content. Seeds, along with developing flowers, also had the highest P content. Our data strongly show that insect herbivores restricted to a single host plant in an agricultural monoculture, forage in a heterogeneous nutritional landscape. By more accurately characterizing the resource base of insect herbivores in terms that are physiologically relevant and at a scale that is ecologically meaningful, this study provides information that is essential for further understanding the nutritional basis of plant-insect interactions.

Introduction

The nutritional component of plant-insect interactions can have a strong influence on insect performance (Simpson and Raubenheimer, 2001; Behmer, Simpson, and Raubenheimer, 2002), community structure, and evolution (Ehrlich and Raven, 1964; Berenbaum, 1983; Jermy, 1984). For insect herbivores, resource quality is primarily determined by the balance of nutrients and defensive chemicals in host plants (Berenbaum, 1995; Hägele and Rowell-Rahier, 1999). Research suggests that many insect species feed to maximize nutrient intake while reducing the consumption of toxins or allelochemicals (Behmer, Raubenheimer, Simpson, 2001; Simpson and Raubenheimer, 2002). Additionally, because insects are capable of assessing concentrations of both nutrients and toxins in their food, nutrient content can impact the

amount of a toxic resource an insect is willing to ingest (Behmer, Raubenheimer, Simpson, 2001; Simpson and Raubenheimer, 2002).

Insects can detect protein (amino acids) and digestible carbohydrates (soluble sugars and starch), and often feed selectively upon specific plant resources based on the concentrations of available nutrients (Schoonhoven, 1968; Bernays and Chapman, 1994; Behmer, 2009). The macronutrient content of an individual's diet can have strong effects on its behavior, performance, growth, survival and fecundity, with the balance, or ratio, of macronutrients and the overall concentration of macronutrients often showing interactive effects (Behmer, Raubenheimer, Simpson, 2001; Simpson et al., 2002; Simpson et al., 2004; Simpson et al., 2006). These nutritional parameters can then influence ecological interactions through population-level effects, such as mass movement (Simpson et al. 2006) and pathogen resistance (Lee et al., 2006; Povey et al., 2008; Cotter et al., 2010; Ponton et al., 2012).

Despite the ecological importance of plant macronutrients, few studies have explicitly measured macronutrient concentrations in plants. Although some protein and carbohydrate data are published for a few agricultural crops (wheat: Stieger and Feller, 1994; legumes: Li at al., 1996; Sánchez et al., 2004), elemental measures such as plant carbon and nitrogen dominate. The prevalence of elemental measures is likely due to the importance of mineral nutrition in plant physiology and the fact that N has historically been used as an indicator of plant quality (Joham, 1951). Elements, however, are difficult to tie directly to insect physiology as insects do not directly metabolize carbon and nitrogen, but rather select resources based on detection of macronutrients and use

these compounds as the biochemical basis of physiological processes (Schoonhoven, 1968; Bernays and Chapman, 1994; Behmer, 2009).

In some cases elemental concentrations have been used to calculate macronutrient content using N-protein conversion factors; however, the conversion factor of 6.25 is often used for all plant material, despite evidence that conversion factors are highly variable across species (Boisen, Bech-Andersen, and Eggum, 1987; Mossé, 1990). This makes the use of generalized conversions inaccurate when more precise protein concentrations are needed, as is often the case in animal nutritional studies. Also, the presence of elements in non-nutritive compounds can further contribute to inaccuracies (Izhaki, 1993; Ezeagu et al., 2002). For instance, plant defensive compounds that contain N, such as alkaloids, can lead to discrepancies in N-protein conversions. In addition, C is found in all organic compounds, including non-nutritive cellulose in plants, making it difficult to develop reliable conversion factors for important physiologically-available macronutrients like digestible carbohydrates. In light of these constraints, elemental measurements are clearly limited for use in accurately determining the composition of plants as nutritional resources for herbivores.

Understanding the physiologically-relevant nutrients that herbivorous insects are getting from their plant resources is paramount to understanding insect nutritional ecology. In addition, the plant itself must be characterized at a scale that is relevant to the herbivore. Most insects are smaller than the plants they consume and feed selectively on specific plant tissues or structures. Many insects feed preferentially on plant reproductive structures, often only ingesting specific parts therein, such as pollen or

seeds. Similarly, many insect larvae feed on only one plant while more mobile adults can move between plants. It is therefore important to account for variation in nutrients across different tissues in individual plants, as well as between individual plants, in order to fully capture the landscape-level nutritional variability that insects experience in the field.

In this study we examine the macronutrient composition of cotton (*Gossypium hirsutum* & *G. barbadense)*, a cosmopolitan agricultural crop which hosts a diverse assemblage of insect herbivores that consume a variety of plant tissues (Bottrell and Adkisson, 1977; Matthews, 1989). The primary tissues of cotton fed upon by herbivores are shown in Figure 2.1 and include (i) terminal growth, which are young leaves, (ii) true leaves, which are larger more mature leaves, (iii) squares, which are developing flowers, and (iv) bolls, which are the fruiting structures. As the plant reaches maturity, cotton squares flower and, upon fertilization, become bolls. Being a more complex structure, bolls also contain three distinct tissues: the rind, or outer green tissue of the boll, the lint, which consists of white fibers, and seeds. In addition to the physiological development of the entire plant, tissues near the base of the plant are older, while those nearer the top of the plant are newly developed and younger. Insect pests such as thrips, armyworms, cutworms, early instar bollworm, aphids, whiteflies, and various beetle species feed on the foliar tissues or phloem of cotton. Plant bugs, fleahoppers, boll weevil larva, and some caterpillar species feed on squares, while stink bugs, armyworms, budworm, and bollworm pierce or bore into cotton bolls, feeding largely on the developing seeds.

The nutritional composition of cotton has largely been inferred from elemental

data (Eaton and Ergle, 1953; Halevy, Marani, and Markovitz, 1987; Mullins and Burmetster, 1990; Fridgen and Varco, 2004), although Showler and Moran (2003) did report soluble protein and carbohydrate content for leaves from plants in different watering regimes. Currently, there is still very little information available on plant macronutrients, particularly at the level of detail that is relevant to insect nutrition (i.e. macronutrient content of different tissues, across plant development, etc.). The objectives of this study were to assess the nutritional variation in cotton as a resource for insect herbivores by explicitly quantifying the concentrations of soluble protein (P) and digestible-carbohydrates (C) in different cotton tissues. First, we measured the P and C content of different tissues across plant development in two *Gossypium hirsutum* (upland cotton) genotypes under greenhouse conditions. Second, we examined how boll size and age impacted macronutrient content of boll-specific tissues, including the rind, lint, and seed, in these same genotypes under greenhouse conditions. Lastly, we looked at tissue macronutrient content in three genotypes of *G. hirsutum* and one genotype of *G. barbadense* (Pima cotton) grown under field conditions. More accurately characterizing the nutritional landscape within and among different tissues, genotypes, and environments provides an ecological framework for parameterizing lab-based nutritional studies, better understanding plant-insect interactions, and exploring proximate and ultimate consequences of insect nutritional ecology.

Methods

Experiment #1 – Greenhouse Conditions

To measure the macronutrient content of different cotton tissues we planted two varieties of upland cotton (*Gossypium hirsutum*) in the greenhouse, a conventional (LA122, All-Tex Seed Co.) and transgenic Bt variety (FM1740B2F, Bayer CropScience). Seeds were planted in potting soil (Metro-Mix 900 Professional Growing Mix) at Texas A&M University in College Station, TX on May 11, 2012,grown to the cotyledon stage in individual planters (72 cell trays), and then transplanted to 7.5 liter pots. All plants were watered with equal amounts as needed.

Eight plants each from both varieties were sampled at three different time points to capture the macronutrient profiles at key physiological stages: (i) 33 days after planting (DAP) before squares were present, (ii) 47 DAP when squares were present, and (iii) 83 DAP when bolls were present. Each plant was cut at soil-level. The node of the first fruiting branch, the number of developing flowers (i.e., squares), and number of fully developed flowers (i.e., bolls) were recorded as measures of development. All terminal growth (newly unfurled leaves smaller than 6 cm in width), true leaves (mature and completely unfurled leaves wider than 6 cm), squares, and bolls were cut from each plant (see Figure 2.1), placed in envelopes, and immediately frozen at -80°C. These tissues were then freeze-dried,weighed, and ground using a Wiley® Mill (Model 3383-L10, 115v, ¼ HP), before being analyzed for soluble protein and digestible carbohydrate content. The remaining stems were placed in an envelope and oven dried at 60°C to

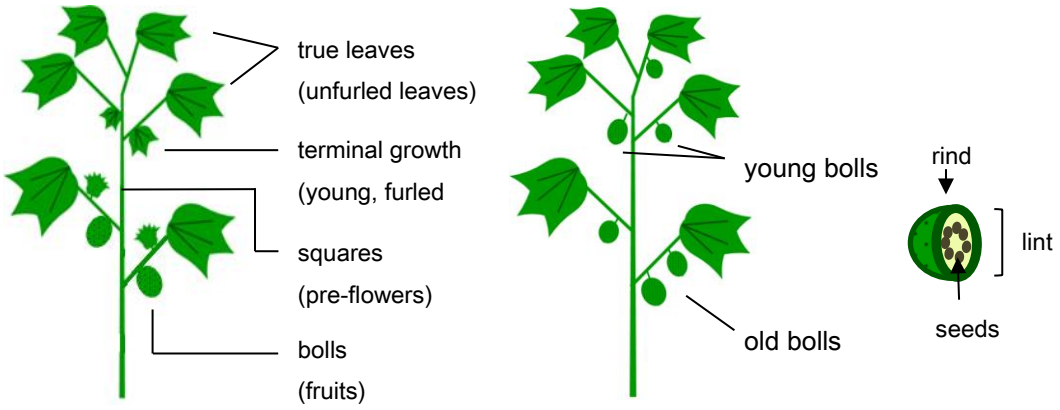

Figure 2.1. A diagram of the different tissues found in cotton, the location of old and young bolls on the plant, and the different tissues found within cotton bolls.

constant mass. After all plant material was dry and weighed, the mass of the sampled tissues were combined with the mass of the stems to determine total aboveground biomass for each plant.

Experiment #2 – Macronutrient Profiles of Differently Aged Bolls

Insects often feed on specific tissues found in more complex plant structures, so in order to understand the finer scale macronutrient dynamics of the more complex cotton boll we examined the macronutrient content of rind, lint, and seed across different boll sizes and ages. All bolls from 15 greenhouse plants were collected at 95 DAP from both conventional (All-Tex LA122) and transgenic cotton (FM1740B3F). Bolls were categorized by their position on the plant (an indicator of tissue age) by dividing each plant into thirds. All bolls taken from the lower third of the plant were classified as "old" and those from the top third as "young" (see Figure 2.1). Within each position, bolls

were then arranged by relative size and divided into “small”, ”medium”, and “large” categories based on the overall range of sizes. Three bolls were randomly selected from each position and size category for each plant and were frozen at -80°C, freeze-dried, weighed, and then dissected. The rind, lint, and seed were separated and weighed. Rind and seed were ground using a Wiley® Mill (Model 3383-L10, 115v, ¼ HP), lint was cut into small pieces by hand, and all tissues were analyzed for soluble protein (P) and digestible carbohydrate content (C).

Experiment #3 – Field Trials

To understand macronutrient dynamics in cotton grown under field conditions, we measured tissue macronutrient content for two different cotton species across four different varieties, or genotypes (all from All-Tex Seed Co.). On April 18th, 2012, one variety (P203) of Pima cotton (*G. barbadense*) and three varieties (LA122, LA1203, and OL220) of upland cotton (*G. hirsutum*) were planted at Texas A&M University AgriLife Research Field Laboratory in Burleson, Co., Tx. The upland varieties included two broadleaf morphotypes (LA122 and LA1203) and one okra-leaf morphotype (OL220). Each plot contained eight 12.2 m rows separated by 1 m, with the outer two rows on each side comprising a buffer and the inner four rows used for data collection. Seeds were planted in either monoculture plots of each variety or quad-culture plots, containing one row of each variety. Monoculture and quad-culture plots were replicated five times, both in a randomize block design across the field.

At 80 DAP, three plants of each variety from every plot were cut at soil-level,

placed in garbage bags, and sealed. In each plot, one plant was collected from each end of the selected row and one from the middle of the row. Because there were different numbers of varieties between monoculture and quad-culture plots, this resulted in 3 plants being taken from each monoculture plot and 12 plants from the quad-culture plots (3 plants per variety). In the monoculture plots the sampled row was randomly selected. In the quad-cultures each row was sampled.

The plants were transported back to the lab and all plants were lightly washed. The number of squares, bolls, and flowers were recorded for each plant. Then, a portion of the terminal growth, true leaves, squares, and bolls were dissected from each plant, pooled, placed into envelopes, and immediately put in a -80°C freezer. All of the terminal growth was harvested, but due to the large size of the mature plants, only 4 true leaves, 4 squares, and 3 bolls were taken from each plant. These tissues were collected from all over the plant, but care was taken to standardize for size across fruiting structures. The collected tissue samples were freeze-dried, ground, weighed, and analyzed for soluble protein (P) and digestible carbohydrates (C) in the same manner as the greenhouse samples. The remainder of the plant was oven dried at 60°C. Once all the plant tissue was dried and weighed, the weights were combined to determine the aboveground biomass for each variety in each plot.

Protein and Carbohydrate Analysis

Approximately 20 mg samples of ground material from each tissue type were used for the soluble protein and digestible carbohydrate assays. Digestible protein

content (all proteins larger than 3000 Daltons) was determined using the Bradford Method, as in Bradford (1976) and Compton and Jones (1985), with alterations from Clissold, Sanson, and Read (2006). Digestible carbohydrates (mono-, oligo-, polysaccharides, as well as methyl derivatives) were quantified using a phenol-sulfuric acid assay, as in Dubois et al. (1956) with alterations from Clissold, Sanson, and Read (2006). All results are represented as the percentage of dry mass. Digestible protein and carbohydrates are hereto referred to in the text as P and C, respectively. The total macronutrient content, which is the combined percentages of P and C (P+C), and the P:C ratio (P/C) were calculated for each tissue at ~80 DAP for both greenhouse and field plants.

Data Analysis

Data were tested for normality. When assumptions of normality could not be met, the data were either ranked-transformed and then re-analyzed or analyzed using a non-parametric Kruskall-Wallis test and/or Mann Whitney-U test. A Tukey HSD was used for all post hoc analyses, unless otherwise specified. All statistics were done using SPSS version 21 for Windows (SPSS Inc., Chicago, IL, USA).

For Experiment 1 (greenhouse conditions), a MANOVA was used to test for the effects of genotype, tissue, and time on P and C content. An ANOVA was used to test the same effects on total macronutrient concentration and P:C ratio. Different tissues were present at different time points, so analyses were performed for each time period separately to determine genotype and tissue effects, while tissues were analyzed

separately to determine genotype and time effects.

For Experiment 2 (bolls only), an ANCOVA with boll dry mass as a covariate was used to determine the main and interactive effects of genotype, boll age, and tissue on boll composition, while controlling for boll size. This was done to determine how the proportion of each boll tissue (by dry mass) varied across genotype and boll age. Similarly, a MANCOVA and ANCOVA were used to determine the main and interactive effects of genotype, boll age, and tissue on P and C content, and total macronutrients and P:C ratio, respectively.

For Experiment 3 (field conditions), a Kruskall-Wallis test was used to determine differences in aboveground dry mass across genotypes. A Bonferroni-corrected Mann Whitney-U test was used for post hoc comparisons. Because there was a significant effect of genotype on aboveground dry mass, plant dry mass was used as a covariate for further analysis. A MANCOVA was used to determine the effect of genotype and tissue on P and C content, while an ANCOVA was used to assess total macronutrient concentration and P:C ratio.

Results

Experiment #1 – Greenhouse Conditions

P and C Content

At 33 DAP, there was no significant effect of genotype or tissue on P and C content, indicating that terminal growth and true leaves (the only tissues present at this time) across both conventional and *Bt* varieties had similar macronutrient profiles (Table

2.1). Table 2.2 shows that these tissues were very P-rich, with P content being over 3 times that of C content. At 47 DAP, when both foliar and reproductive tissues were present, there was a significant effect of tissue on P and C content, which univariate results showed was due to significant differences in both P and C content (Table 2.S1). Figure 2.2 and the individual contrasts in Table 2.1 show that terminal growth and true leaves had similar profiles, but were different from squares, which had significantly lower P content than both terminal growth and true leaves (see Table 2.2). A significant tissue effect was also seen at 83 DAP, which was again the result of significant differences in both P and C content (Table 2.S1). All tissues except for terminal growth/ true leaves and true leaves/squares had distinct macronutrient profiles (Table 2.1). Figure 2.2 shows that there was a sharp divide between the foliar tissues and reproductive

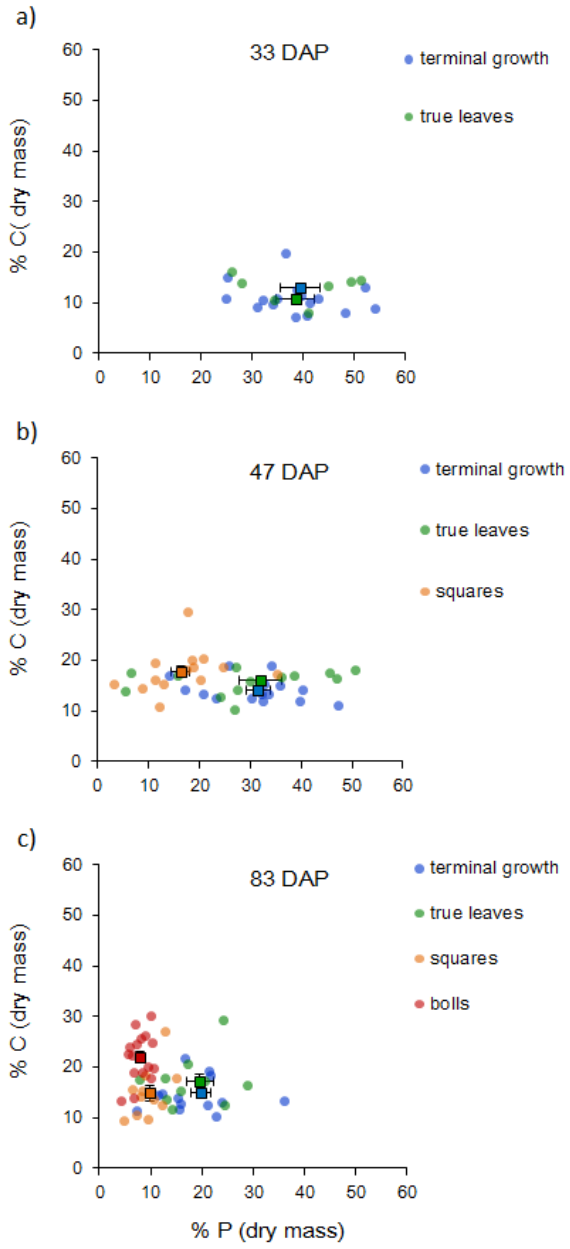

Figure 2.2. A scatterplot of the P and C content (circles) for different cotton tissues in greenhouse plants (both genotypes pooled), and the average (squares) for each tissue, at (a) 33 DAP, (b) 47 DAP, and (c) 83 DAP.

tissues in terms of P content, with terminal growth and true leaves containing significantly more P than squares and bolls (Table 2.2). For C content, bolls had a higher percentage than all other tissues, except true leaves. There were also significant changes in P and C content over time for terminal growth (Pillai's Trace, $F_{4,67}$=8.51, P<0.0001) and true leaves (Pillai's Trace, $F_{4,52}$=4.18, P=0.005). In both tissues, C content increased over time while P content decreased (Figure 2.2).

Total Macronutrients

In terms of total macronutrients, Table 2.3 shows there was no genotype or tissue effect at 33 DAP. Table 2.2 shows that P and C made up about 50% of the total dry mass in terminal growth and true leaves at this time. At 47 DAP there were strong differences between tissues, with terminal growth and true leaves containing approximately %15 more total macronutrients than squares (Table 2.2). At 83 DAP, there were again no genotype or tissue effects, indicating that all tissues had the same total macronutrient content. Figure 2.2 shows that there was a significant decrease over time in macronutrient concentration in both terminal growth (F_2=15.23, P<0.0001) and true leaves (F_2=4.55, P=0.019). For squares, there was a time*genotype interaction (F_1=6.77, P=0.016) across the last two sampling periods. Square total macronutrient content increased over time in the conventional (LA122) genotype and decreased over time in the *Bt* (FM1740B2F) genotype.

Table 2.1. MANOVA results (Pillai's trace) for greenhouse plants at each time period, as well as the individual contrasts across tissues. A strict Bonferroni correction was used for multiple comparisons (critical α value for 47 DAP was P=0.0167 and 83 DAP is P=0.0083). Bolded values indicate significance.

Time	Variable	Source	df	F-ratio	P-value
a) 33 DAP	P and C	genotype	2	0.563	0.579
		tissue	2	0.983	0.393
		genotype*tissue	2	1.911	0.177
b) 47 DAP	P and C	genotype	2	0.305	0.739
		tissue	4	5.197	**0.001**
		genotype*tissue	4	1.102	0.362
	Individual contrasts				
	terminal growth *vs.* true leaves		2	1.428	0.252
	terminal growth *vs.* squares		2	12.144	**<0.000**
	true leaves *vs.* squares		2	6.365	**0.004**
c) 83 DAP	P and C	genotype	2	2.042	0.143
		tissue	6	8.747	**<0.000**
		genotype*tissue	6	1.991	0.076
	Individual contrasts				
	terminal growth *vs.* true leaves		2	1.183	0.316
	terminal growth *vs.* squares		2	7.893	**0.001**
	terminal growth *vs.* bolls		2	36.720	**<0.000**
	true leaves *vs.* squares		2	5.068	0.010
	true leaves *vs.* bolls		2	21.212	**<0.000**
	squares *vs.* bolls		2	8.054	**0.001**

P:C Ratio

Table 2.3 shows that there was no effect of genotype or tissue on P:C ratio at 33 DAP. At this time period, terminal growth and true leaves were extremely P-biased (Table 2.2). At 47 DAP, a significant tissue effect was apparent, with terminal growth and true leaves displaying a P:C ratio more than double that of squares (Table 2.2). There was also a significant tissue effect at 83 DAP. All tissues had distinct P:C ratios

except for terminal growth/true leaves and true leaves/squares, which were statistically similar. True leaves and terminal growth had the highest, most P-biased ratios, while squares and bolls displayed C-biased ratios. Figure 2.2 shows that time also had a significant impact on P:C ratio for both true leaves (F_2=7.14, P=0.003) and terminal growth (F_2=22.54, P<0.0001). In both cases, tissue P:C decreased steadily from 33 to 83 DAP, resulting in foliar tissues that were more C-biased as the plant aged.

Experiment #2 – Macronutrient Profiles of Differently Aged Bolls

Boll Composition

There was a significant effect of tissue and a tissue*age interaction on boll composition for the greenhouse plants (Table 2.4). The proportions of rind, lint, and seed were equal in old bolls; however, rind proportions were much higher than that of lint and seed in young bolls (Figure 2.3). Table 2.4 also shows that age had unique effects on these tissues. As bolls aged, rind proportions decreased, while lint and seed proportions increased, likely due to changes in the surface area-to-volume ratio of the bolls (Figure 2.3).

P and C Content

Although P and C content varied with several factors, overall, the lint showed the lowest P and C content, followed by rind. Seed, on the other hand, had considerably higher P and C, at as much as 60 times the amount of P and 10 times of the amount of C found in the other tissues. Table 2.5 shows that there was a significant

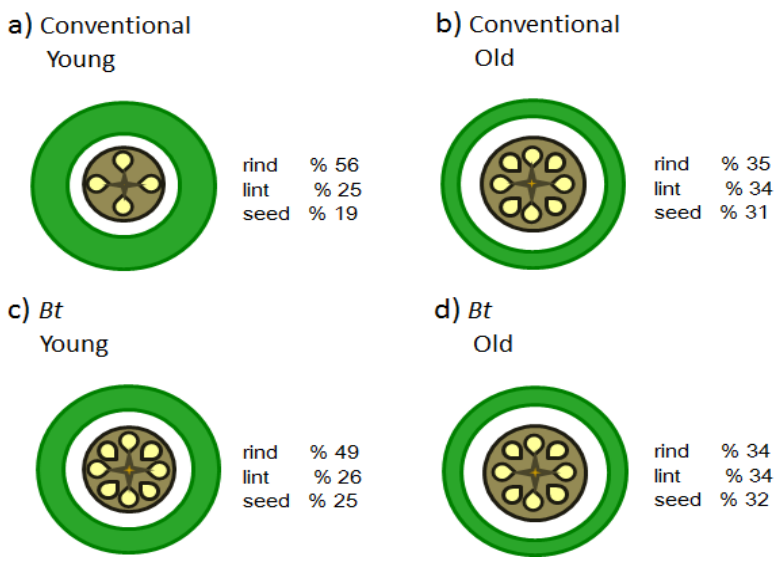

Figure 2.3. A diagram showing the relative composition of different boll tissues across genotype and boll age. The area of the colored portions display the average percentage of total dry mass accounted for by rind (green), lint (white), and seed (brown) for greenhouse bolls collected at 95 DAP.

genotype*age*tissue interaction for greenhouse bolls; however, this effect was due to differences in P content only. Genotype had a significant effect on P content in rind tissue only, with the conventional (LA122) genotype showing higher rind P content than the *Bt* (FM1740B3F) genotype for both young and old bolls (Table 2.S2). In both genotypes, differences in tissue P content were only significant in older bolls, as the P content of rind, lint, and seed in younger bolls were all similar (Table 2.S2). For the conventional genotype, all tissues in old bolls were distinct, with seeds showing the highest P content, followed by rind, and then lint. For the *Bt* genotype, rind and lint showed similar P content, while seed again had significantly higher P concentrations. Boll age had different impacts across tissues (Table 2.S2). There was no effect of boll age on rind in the conventional genotype; however, for the *Bt* variety, rind P content was much higher in the younger bolls. For both varieties, lint P content was greater in

younger bolls, while seed P content was greater in older bolls. Exact P and C percentages across genotype, tissue, and boll age are shown in Table 2.6.

Table 2.2. The average P and C content, total macronutrients, and P:C ratio for each tissue type in greenhouse plants at each sampling period and field plants at 80 DAP.

Time	Tissue	% P	% C	Total	P:C
33 DAP	terminal	38.5 ± 2.11	10.9 ± 0.78	49.4 ± 2.1	3.83 ± 0.35
	true leaves	39.4 ± 3.82	12.9 ± 0.84	52.2 ± 3.86	3.24 ± 0.44
	squares	-	-	-	-
	bolls	-	-	-	-
47 DAP	terminal	31.5 ± 2.32	14.1 ± 0.63	44.8 ± 2.06	2.27 ± 0.23
	true leaves	32.1 ± 4.22	16.1 ± 0.57	47.9 ± 4.43	2.02 ± 0.25
	squares	16.3 ± 1.97	17.8 ± 1.05	32.9 ± 2.93	0.949 ± 0.12
	bolls	-	-	-	-
83 DAP	terminal	19.7 ± 1.89	14.8 ± 1.00	32.6 ± 2.11	1.35 ± 0.17
	true leaves	19.5 ± 2.62	17.3 ± 1.32	34.5 ± 2.89	1.11 ± 0.15
	squares	9.8 ± 0.85	14.8 ± 1.51	24.4 ± 2.13	0.672 ± 0.06
	bolls	7.9 ± 0.48	21.9 ± 1.20	29.8 ± 1.42	0.37 ± 0.03
Field	terminal	23.5 ± 0.69	18.8 ± 0.35	42.7 ± 0.72	1.29 ± 0.05
	true leaves	16.1 ± 0.76	9.33 ± 0.14	24.8 ± 0.80	1.67 ± 0.09
	squares	16.1 ± 0.45	1.1 ± 0.02	17.4 ± 0.49	14.7 ± 0.47
	bolls	11.4 ± 0.51	29.9 ± 0.36	41.6 ± 0.73	0.39 ± 0.02

Tissue Macronutrients

Table 2.7 shows that there was a significant effect of genotype and an age*tissue interaction for total macronutrients in bolls. Across tissues and boll age, the conventional genotype had significantly higher total macronutrient content than the *Bt* genotype (Table 2.6). Across genotypes, total macronutrients decreased in rind and lint as bolls aged; however, seed macronutrient content increased with boll age. In old bolls, all

Table 2.3. ANOVA results for total macronutrients and P:C ratio for greenhouse plants at each time period. Tukey's HSD was used for all post hoc analyses.

Time	Variable	Source	df	F-ratio	P-value
a) 33 DAP	total macronutrients	genotype	1	0.862	0.365
		tissue	1	0.695	0.415
		genotype*tissue	1	1.198	0.287
	P:C	genotype	1	0.677	0.421
		tissue	1	0.585	0.454
		genotype*tissue	1	3.593	0.073
b) 47 DAP	total macronutrients	genotype	1	0.325	0.572
		tissue	2	6.203	**0.005**
		genotype*tissue	2	1.333	0.275
	Post hoc results				
	terminal growth *vs.* true leaves				0.999
	terminal growth *vs.* squares				**0.033**
	true leaves *vs.* squares				**0.005**
	P:C	genotype	1	0.695	0.410
		tissue	2	10.572	**<0.000**
		genotype*tissue	2	0.488	0.618
	Post hoc results				
	terminal growth *vs.* true leaves				0.970
	terminal growth *vs.* squares				**<0.000**
	true leaves *vs.* squares				**0.004**
c) 83 DAP	total macronutrients	genotype	1	1.530	0.222
		tissue	3	2.281	0.092
		genotype*tissue	3	2.228	0.098
	P:C	genotype	1	0.484	0.490
		tissue	3	29.025	**<0.000**
		genotype*tissue	3	0.270	0.847
	Post hoc results				
	terminal growth *vs.* true leaves				0.999
	terminal growth *vs.* squares				**0.003**
	terminal growth *vs.* bolls				**<0.000**
	true leaves *vs.* squares				0.142
	true leaves *vs.* bolls				**<0.0001**
	squares *vs.* bolls				**0.002**

tissues were distinct, with lint having the lowest macronutrient content, followed by rind, and then seed (Table 2.S3). In young bolls, however, rind and lint had similar macronutrient content, which were again significantly lower than that of seed (Table 2.6).

Table 2.4. ANCOVA results (boll mass as a covariate) for boll tissue composition in greenhouse plants at 95 DAP. Tukey's HSD was used for all post hoc analyses.

Variable	Source		df	F-ratio	P-value
tissue %	boll mass		1	0.947	0.333
	genotype		1	0.10	0.920
	age		1	0.266	0.608
	tissue		2	30.35	**<0.000**
	genotype*age		1	1.153	0.286
	genotype*tissue		2	1.660	0.196
	age*tissue		2	23.92	**<0.000**
	genotype*age*tissue		2	1.294	0.279
Post hoc results					
	rind	old *vs.* young			**<0.000**
	lint	old *vs.* young			**0.006**
	seed	old *vs.* young			**0.002**
	old	rind *vs.* lint			0.999
		rind *vs.* seed			0.999
		lint *vs.* seed			0.980
	young	rind *vs.* lint			**<0.000**
		rind *vs.* seed			**<0.000**
		lint *vs.* seed			0.603

Table 2.5. MANCOVA (Pillai's trace, dry mass as a covariate) and univariate results for P and C across boll tissues and genotypes in greenhouse plants at 95 DAP and contrasts. Bolded values indicate significance.

Variable	Source	df	F-ratio	P-value
P and C	boll mass	2	3.023	0.054
	genotype	2	8.202	**0.001**
	age	2	34.68	<**0.000**
	tissue	4	31.54	<**0.000**
	genotype*age	2	1.505	0.228
	genotype*tissue	4	1.616	0.173
	age*tissue	4	15.05	<**0.000**
	genotype*age*tissue	4	2.581	**0.039**
Univariate Results				
P	genotype*age*tissue	2	3.567	**0.033**
C	genotype*age*tissue	2	1.968	0.146

Table 2.6. P and C percentages across genotype, position, and tissue for greenhouse bolls at 95 DAP.

Genotype	Tissue	Boll Age	% P	% C	% P+C	P:C
con	rind	old	7.80 ± 0.70	10.2 ± 0.50	18.1 ± 0.95	0.772 ± 0.07
		young	8.63 ± 0.70	17.1 ± 1.45	25.8 ± 1.54	0.525 ± 0.06
	lint	old	1.54 ± 0.71	3.08 ± 0.72	4.62 ± 0.13	0.453 ± 0.10
		young	10.8 ± 5.81	23.3 ± 3.39	34.2 ± 8.09	0.404 ± 0.17
	seed	old	59.2 ± 4.94	19.5 ± 1.13	78.7 ± 4.18	3.22 ± 0.39
		young	21.2 ± 4.40	33.6 ± 1.95	54.8 ± 3.35	0.687 ± 0.20
Bt	rind	old	1.62 ± 0.67	9.50 ± 0.93	11.16 ±1.22	0.181 ± 0.06
		young	5.77 ± 0.65	16.2 ± 1.44	21.9 ± 1.90	0.359 ± 0.03
	lint	old	0.79 ± 0.24	5.38 ± 2.83	7.18 ± 3.75	0.253 ± 0.08
		young	5.53 ± 3.26	17.2 ± 1.61	22.5 ± 3.46	0.372 ± 0.25
	seed	old	60.7 ± 5.26	20.0 ± 2.12	80.8 ± 3.94	3.35 ± 0.42
		young	19.9 ± 3.93	33.3 ± 2.51	53.3 ± 3.77	0.656 ± 0.17

Table 2.7. ANCOVA (boll dry mass as a covariate) results for total macronutrient content and P:C ratio in greenhouse bolls collected at 95 DAP.

Variable	Source	df	F-ratio	P-value
total macronutrients	boll mass	1	2.23	0.139
	genotype	1	4.48	**0.037**
	age	1	23.23	**<0.000**
	tissue	2	208.9	**<0.000**
	genotype*age	1	0.583	0.447
	genotype*tissue	2	2.35	0.102
	age*tissue	2	49.71	**<0.000**
	genotype*age*tissue	2	2.60	0.081
P:C	boll mass	1	0.274	0.602
	genotype	1	19.93	**<0.000**
	age	1	7.30	**0.008**
	tissue	2	33.81	**<0.000**
	genotype*age	1	1.47	0.228
	genotype*tissue	2	2.86	0.063
	age*tissue	2	15.39	**<0.000**
	genotype*age*tissue	2	2.58	0.082

P:C Ratio

Table 2.7 shows there was a significant effect of genotype and an age*genotype interaction for P:C ratio in bolls. Across tissues and boll age, the conventional genotype had a higher, more P-biased, ratio than the *Bt* genotype (Table 2.6). Across genotypes, seed was the only tissue that was significantly impacted by boll age (Table 2.S3), with older bolls containing seed that was more P-biased than the seed in younger bolls (Table 2.6). In older bolls, rind and lint had a similar P:C ratio, while seed was significantly more P-biased (Table 2.S3). In younger bolls, however, the P:C ratio of seed was similar to that of rind and only significantly greater than lint (Table 2.S3). Overall, all three

tissues were C-biased, except for seed, which was extremely P-biased in old bolls only (Table 2.6).

Experiment #3 – Field Trials

Varieties

There were significant differences in average plant aboveground dry mass across varieties in the field ($H=45.3$, $df=3$, $p<0.0001$). Figure 2.4 shows that P203 (Pima cotton) had the highest average dry mass at 24.26 g (± 0.874), followed by LA1203 at 20.82 g (± 0.805). LA122 and OL200 had statistically similar dry mass ($U=608$, $z= -1.167$, $P=.243$) at 17.63 g (± 0.843) and 16.79 g (± 0.952) respectively. Due to these significant differences, biomass was used as a covariate for further macronutrient analyses.

P and C Content

Table 2.8 shows a significant genotype and tissue effect was apparent for field plants. Contrasts indicate that only P203 and LA1203 genotypes had significantly different profiles; however, univariate results, shown in Table 2.S4, indicate that this was only due to differences in C content. Figure 2.4a shows that P203 plants had significantly lower C content than the other genotypes. All tissues had significantly distinct P and C profiles, which resulted from differences in both P and C content (Table 2.S4). Figure 2.4b shows that terminal growth had the highest P content, followed by true leaves and squares, which were intermediate, and bolls with the lowest P content. In

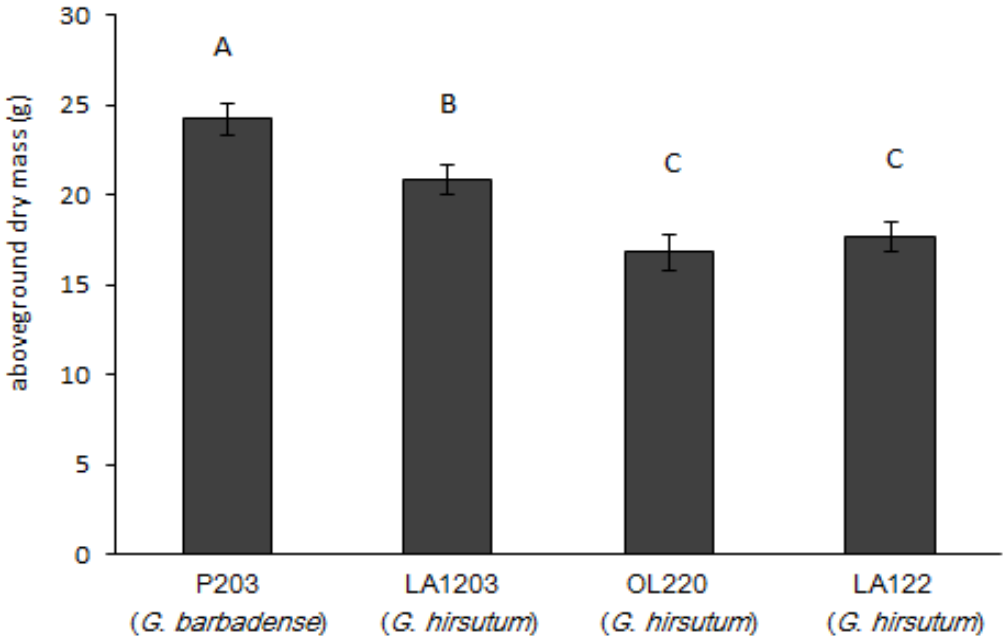

Figure 2.4. The average aboveground dry mass for each field genotype. Error bars show 1± SE and different letters indicate significant differences at

terms of C content, all tissues were distinct, with bolls having the highest content, followed by terminal growth, true leaves, and squares with the lowest C content (Table 2.2).

Total Macronutrients

Table 2.9 shows that there was a significant tissue effect on total macronutrient content for field plants. Figure 2.4b shows that terminal growth and bolls were similar and had the highest total macronutrient concentrations, followed by true leaves, and squares with the lowest (Table 2.2).

Table 2.8. MANCOVA results (Pillai's trace, dry mass as a covariate) for field plants and contrasts between genotype and tissue. A Bonferroni correction was used for multiple comparisons (critical value P=0.0083). Bolded values indicate significance.

Variable	Source	df	F-ratio	P-value
P and C	dry mass	2	0.873	0.420
	genotype	6	3.085	**0.006**
	tissue	6	132.3	**<0.000**
	genotype*tissue	18	1.141	0.311
variety				
Individual contrasts				
P203 *vs.* LA122		2	3.750	0.025
P203 *vs.* LA1203		2	8.077	**<0.001**
P203 *vs.* OL220		2	2.426	0.091
LA122 *vs.* LA1203		2	0.873	0.419
LA122 *vs.* OL220		2	0.477	0.621
LA1203 *vs.* OL220		2	2.870	0.059
tissue				
Individual contrasts				
terminal growth *vs.* true leaves		2	177.9	**<0.000**
terminal growth *vs.* squares		2	542.0	**<0.000**
terminal growth *vs.* bolls		2	238.9	**<0.000**
true leaves *vs.* squares		2	120.2	**<0.000**
true leaves *vs.* bolls		2	585.7	**<0.000**
squares *vs.* bolls		2	1189	**<0.000**

Table 2.9. ANCOVA results (dry mass as a covariate) for total macronutrients and P:C ratio, as well as individual contrasts, for field plants at 80 DAP. Tukey's HSD was used for all post hoc analyses.

Variable	Source	df	F-ratio	P-value
total macronutrients	dry mass	1	0.438	0.509
	genotype	3	1.893	0.133
	tissue	3	194.6	**<0.000**
	genotype*tissue	9	1.464	0.166
Individual contrasts				
terminal growth *vs.* true leaves				**<0.000**
terminal growth *vs.* squares				**<0.000**
terminal growth *vs.* bolls				0.999
true leaves *vs.* squares				**<0.000**
true leaves *vs.* bolls				**<0.000**
squares *vs.* bolls				**<0.000**
P:C	dry mass	1	0.281	0.597
	genotype	3	1.337	0.264
	tissue	3	293.7	**<0.000**
	genotype*tissue	9	0.910	0.518
Individual contrasts				
terminal growth *vs.* true leaves				**<0.000**
terminal growth *vs.* squares				**<0.000**
terminal growth *vs.* bolls				**<0.000**
true leaves *vs.* squares				**<0.000**
true leaves *vs.* bolls				**<0.000**
squares *vs.* bolls				**<0.000**

P:C Ratio

Table 2.9 shows that there was a significant tissue effect on P:C ratio in field plants. As seen in Figure 2.4b, all of the tissues showed distinct P:C ratios. Terminal growth was the most C-biased tissue, having the lowest average ratio, followed by true

leaves, then bolls. Squares, however, were extremely P-biased displaying a ratio almost 12 times that of the lowest ratio exhibited by bolls (Table 2.2).

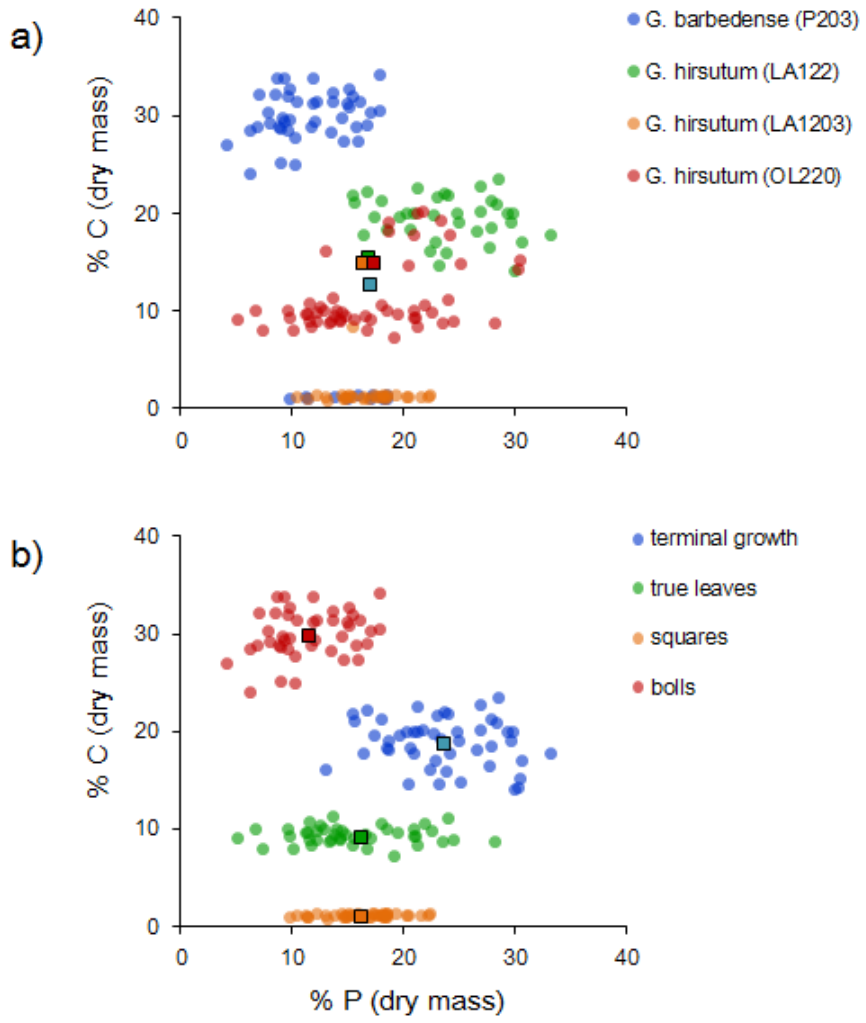

Figure 2.5. A scatterplot of the P and C content (circles) and average (squares) for (a) each field genotype and (b) each tissue type at 80 DAP. Dotted lines indicate total macronutrient content ranges. Error bars show 1± SE.

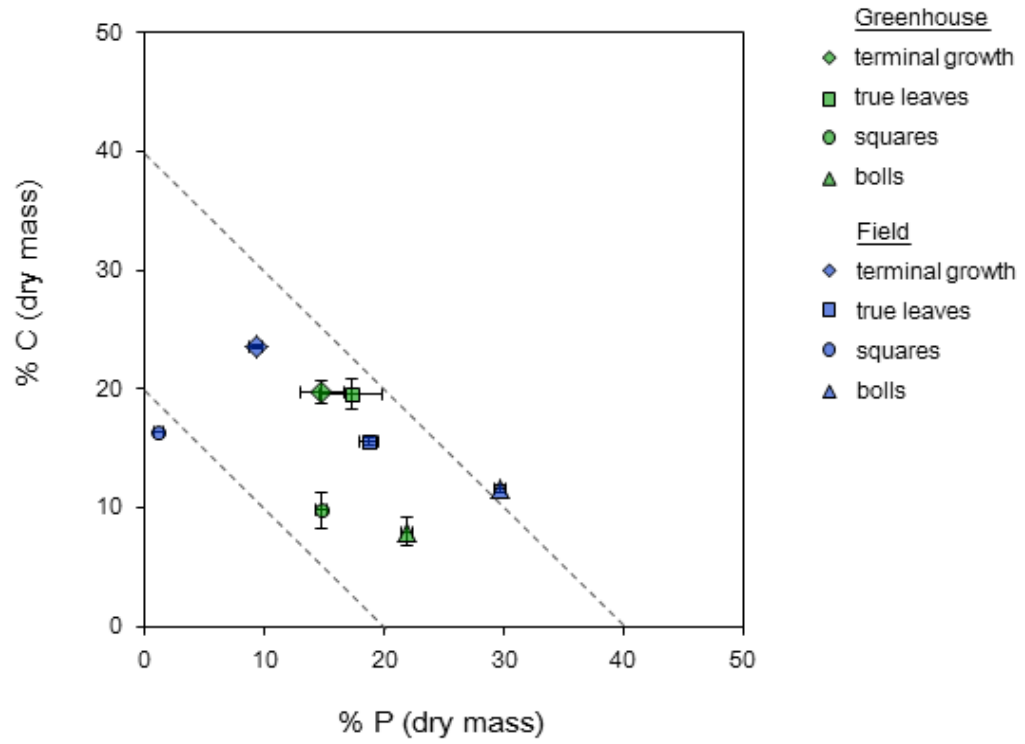

Figure 2. 6. The average P and C content for each tissue in greenhouse (83 DAP) and field (80 DAP) plants. Dashed lines indicate total macronutrient concentrations at 20% and 40%. Error bars show 1± SE.

Discussion

The objectives of this study were to characterize cotton as a nutritional resource for insect herbivores by measuring available protein and carbohydrate content, total macronutrient concentrations, and P:C ratios at several levels of organization: (1) within individual plants, both spatially (across tissues) and temporally (across plant age), (2) across different genotypes, and (3) across different growing environments. This was done to determine the overall concentrations and relative proportions of macronutrients

available to insect herbivores, and also to document the level of macronutrient variability experienced by insect herbivores in natural environments.

Overall, our data show that even in an agricultural monoculture, such as a cotton field, insects are foraging in a highly heterogeneous nutritional landscape. P and C content, total macronutrients, and P:C ratio varied significantly across tissue type, plant age, genotype, and environment. We also found evidence that within complex tissues, such as bolls, there is strong nutrient compartmentalization and fine-scale nutrient dynamics.

The strongest contrasts observed were between cotton tissues. In both greenhouse and field environments, we found over a 2-fold variation in total macronutrient content between different tissues. Protein content was relatively stable in comparison to C, varying only 2.8-fold across tissue type; however, C content was highly variable, with content fluctuating over 27-fold between bolls (highest) and squares (lowest) in the field. This variability produced a 40-fold difference in P:C ratio between tissues, indicating that more variability exists in the proportion of macronutrients than in total concentrations across tissues.

This variability likely has strong physiological and ecological repercussions for insect herbivores feeding in plant systems. Across a diversity of insect herbivore taxa, studies have shown that diet macronutrient content, particularly P and C concentrations and ratios, have significant impacts on insect performance and life history, including survival, mass gain, fecundity, and developmental time (Simpson and Raubenheimer 1995, 2003; Lee et al., 2002; Behmer, 2009; Le Gall and Behmer, 2014; Roeder and

Behmer, 2014), as well as immunity (Lee et al., 2006; Lee, Simpson, and Wilson, 2008; Povey et al., 2008; Cotter et al., 2010) and detoxification ability (Raubenheimer, 1992; Simpson and Raubenheimer, 2001; Behmer, Simpson, and Raubenheimer, 2002).

Specifically in cotton, Celorio-Mancera et al. (2012) found that feeding larvae on different cotton tissues had strong effects on mass gain and gene expression in the Old World bollworm, *Helicoverpa armigera*. They used gut gene expression profiles from *H. armigera* to perform a hierarchical clustering analysis and showed that the expressional responses from larvae feeding on cotton leaves and squares, which our data show to have similar macronutrient profiles, clustered together, while bolls were a separate group. The correspondence between our tissue macronutrient data and the performance and transcriptional results from Celorio-Mancera et al. (2012) provide more compelling evidence that variability in plant macronutrients has strong physiological impacts on herbivores.

In this study, we also saw significant variability in tissue macronutrients over time, with P generally decreasing and C content increasing or staying the same in the all tissues throughout the growing season. These changes over the growing season suggest that there may be a connection between plant phenology and macronutrient content, which has the potential to impact the temporal abundance of herbivore species, as well as their movement between different tissue types and host plant species.

In the early-season, insects such as thrips, aphids, and cutworms are commonly found in cotton, as young foliar tissue likely represents a nutritious resource. Although we did not explicitly measure the macronutrient content of cotton seedlings, our data do

show that terminal growth and true leaves had the highest macronutrient concentrations at 15 DAP. This suggests that young cotton tissues are a highly nutritious resource for early season herbivores. Because we did not assay macronutrient concentrations in the xylem or phloem (including free amino acid concentrations), our data cannot make precise predictions about resource quality for sucking bugs such as phloem-feeding aphids. That being said, high nutrient allocation to young growing tissues strongly suggests high nutrient concentrations are being transported in the plant vascular system (Hewitt and Smith, 1974). It should also be noted that secondary compounds, such as gossypol and its derivatives, tend to be lower in younger tissue (Bell, 1986), which may also influence herbivore preference in addition to nutritional considerations (Behmer, Raubenheimer, Simpson, 2001; Simpson and Raubenheimer, 2002).

Mid-season colonizers, such as plant bugs and bollworms, likely encounter the greatest nutritional diversity, as they typically forage in cotton when all tissues assayed here are simultaneously present. Bollworm adults typically lay eggs on cotton squares and, once hatched, larvae tend to feed initially on terminal growth but later on the squares and seeds of developing bolls (Boyd, Phipps, and Wrather, 2004; Quaintance and Brues, 1905). Bollworms have been documented to feed on virtually all cotton tissues, targeting foliar tissues during early larval instars and reproductive tissues during later instars (Boyd, Phipps, and Wrather, 2004; Quaintance and Brues, 1905).

Our field data show that terminal growth and squares are comparable in terms of total macronutrients and P, whereas seeds are much higher in both. Observed feeding patterns suggest that bollworms foraging in cotton tend to target high macronutrient

tissues in early life stages, but prioritize P as they develop, which is also supported by nutritional studies showing that bollworm prefer P-biased diets (Waldbauer and Cohen, 1984; Deans et al., *submitted*). There is also evidence that later instars of *Heliothis* species, which are closely related to bollworm and have similar macronutrient preferences, feed selectively on older bolls (Wilson and Waite, 1982), which this study shows to have greater seed mass and higher seed P content than younger bolls.

Interestingly, there is evidence that bollworm feed differently on genetically-modified *Bt* plants. Gore et al. (2002) showed that larvae on *Bt* plants tended to feed more often near unfertilized flowers. The expression of Cry toxins produced in these plants does vary across tissues (Greenplate, 1999; Adamczyk et al., 2001), and a follow-up study by Gore et al. (2005) suggested that this behavior was primarily due to larvae trying to avoid tissues with high Cry expression; however, there may also be a nutritional component to this behavior. Studies have shown that insects challenged with plant allelochemicals have been documented to prefer high-P diets (Behmer, Raubenheimer, Simpson, 2001; Simpson and Raubenheimer, 2002), and as unfertilized flowers (essentially squares in our analysis) were found to be a P-rich resource in this study, it is also possible these larvae were selecting tissues that were nutritionally optimal for detoxification.

Like bollworm, *Lygus* nymphs and adults also primarily feed on squares and small bolls (Wilson, et al., 1984; Leigh et al., 1988; Greene et al., 1999). Their general feeding ecology and movement patterns are not well understood; however, there is some preliminary evidence suggesting that they also prefer P-biased resources (Deans et al., *in*

prep), which does correspond to the high P concentrations found in squares and developing seeds in bolls under field conditions.

Insects that feed on cotton later in the season, such as stink bugs and leaf-footed bugs, tend to specialize on developing seeds. Our data show that seeds have the highest concentration of total macronutrients and a high P content late in the season. Also, despite finding decreases in P content throughout the season in all other tissues, seed mass and P content actually increases as bolls age, which likely makes the seeds within late-season bolls a highly nutritious and abundant resource.

In this study, we also observed a strong effect of growing environment on plant macronutrients, which reflects the dynamic nature of plant nutrient allocation patterns. Overall, P content was much more stable in the field than the greenhouse, while C content varied more drastically in the field. Also, some tissues showed more variability than others. At 80 DAP, the C content of squares dropped from an average of 14.7% in the greenhouse to 1.1% in the field, while foliar tissues varied less than 5% between environments. These large discrepancies between greenhouse and field plants may have important implications for insect studies which utilize greenhouse plants to test field-relevant interactions, as differences in insect nutrition may lead to confounds. The cause for these fluctuations is not yet clear; however, differences in abiotic factors, such as light intensity, wind, water and nutrient availability, as well as biotic interactions with soil microbes, are likely candidates. Ultimately, future research in identifying the connections between these abiotic factors and macronutrient fluxes will greatly improve our understanding of nutrient relations between plants and insects.

A final conclusion of this study is that attention to scale is extremely important for accurately describing resource quality. Although we found whole intact bolls to contain the lowest P concentrations and the highest C concentrations, when we took a closer look we found strong compartmentalization of nutrients in specific boll tissues. While it was initially surprising that bolls appeared to be a low quality resource (very C-biased), given that many insects preferentially feed on fruiting structures, we ultimately found that the seed contained within the bolls were the most nutritious of all the tissues measured. In light of this, it may be necessary for future research to focus more on specific tissues within plant structures to accurately characterize the nutritional composition of what insect herbivores are actually ingesting.

This study is the first to measure plant macronutrient content on different spatial and temporal scales, by simultaneously characterizing macronutrient content at the individual level (across plant tissues), at the genotypic level (across varieties), and at the environmental level (greenhouse and field environments). Our results indicate that plant macronutrient content can potentially account for a large portion of the environmental variability in nutrients encountered by insects in natural systems. These data, and future macronutrient surveys in other plant systems, will perhaps be most useful for informing laboratory studies exploring the connections between nutrition and herbivore behavior and/or performance that are relevant to the field conditions. Laboratory studies, particularly those employing the geometric framework approach to nutrition (Simpson and Raubenheimer, 1995), have already been incredibly useful for delineating insect nutritional requirements and preferences, yet there has been less progress in

characterizing the actual nutritional landscapes in which the insects forage. In particular, data on the concentrations and variability of macronutrients, rather than simple elemental measures, in natural or agricultural plant communities are severely lacking. The results of this study show that nutritional variability is apparent, even in a seemingly homogeneous environment like a cotton monoculture, and understanding how this variability impacts plant-insect interactions will have important implications for a range of biological fields, from insect physiology and behavioral ecology to agroecology and pest management.

Acknowledgements

We would like to thank all of those who have contributed to this project, either through assistance with field collection, chemical analyses, or general feedback, including: Paul Lenhart, Marion Le Gall, Rebecca Clark, Fiona Clissold, Mikey Eubanks, Cesar Valencia, Lauren Kalns, Diana Castillo-Lopez, Julissa Elk-Ramos, Nicole Locke, and Steve Hague's lab.

Supplemental Tables/Figures

Table 2.S1. Univariate results for P and C content in greenhouse plants.

Time	Variable	Source	df	F-ratio	P-value
a) 47 DAP	P	tissue	2	6.821	0.003
	Individual contrasts				
	terminal growth *vs.* true leaves				0.999
	terminal growth *vs.* squares				**0.005**
	true leaves *vs.* squares				**0.014**
	C	tissue	2	4.892	0.013
	Individual contrasts				
	terminal growth *vs.* true leaves				0.366
	terminal growth *vs.* squares				**0.010**
	true leaves *vs.* squares				0.368
b) 83 DAP	P	tissue	3	19.376	**<0.000**
	Individual contrasts				
	terminal growth *vs.* true leaves				0.999
	terminal growth *vs.* squares				**0.003**
	terminal growth *vs.* bolls				**<0.000**
	true leaves *vs.* squares				**0.015**
	true leaves *vs.* bolls				**<0.000**
	squares *vs.* bolls				0.257
	C	tissue	3	6.272	**0.001**
	Individual contrasts				
	terminal growth *vs.* true leaves				0.894
	terminal growth *vs.* squares				0.999
	terminal growth *vs.* bolls				**0.001**
	true leaves *vs.* squares				0.999
	true leaves *vs.* bolls				0.191
	squares *vs.* bolls				**0.024**

Table 2.S2. Post hoc results for the genotype*age*tissue interaction for P content in greenhouse bolls from 95 DAP. Tukey's HSD was used for all post hoc analyses.

Variables		Contrasts	P-value
con	rind	high vs low	0.942
	lint	high vs low	**<0.000**
	seed	high vs low	**0.007**
Bt	rind	high vs low	**<0.000**
	lint	high vs low	**0.002**
	seed	high vs low	**<0.000**
rind	low	con vs Bt	**<0.000**
	high	con vs Bt	0.343
lint	low	con vs Bt	0.194
	high	con vs Bt	**0.028**
seed	low	con vs Bt	0.951
	high	con vs Bt	0.476
low	con	rind vs lint	**<0.000**
		rind vs seed	**<0.000**
		lint vs seed	**<0.000**
low	Bt	rind vs lint	0.322
		rind vs seed	**<0.000**
		lint vs seed	**<0.000**
high	con	rind vs lint	.999
		rind vs seed	0.256
		lint vs seed	0.065
high	Bt	rind vs lint	0.156
		rind vs seed	0.114
		lint vs seed	**<0.000**

Table 2.S3. Post hoc results for the age*tissue interaction for total macronutrients and P:C ratio in the greenhouse bolls collected at 95 DAP. Tukey's HSD was used for all post hoc analyses.

Variables		Contrasts	P-value
total macronutrients	old	rind *vs* lint	**<0.000**
		rind *vs* seed	**<0.000**
		lint *vs* seed	**<0.000**
	young	rind *vs* lint	0.743
		rind *vs* seed	**<0.000**
		lint *vs* seed	**<0.000**
	rind	old *vs* young	**<0.000**
	lint	old *vs* young	**<0.000**
	seed	old *vs* young	**<0.000**
P:C	old	rind *vs* lint	0.537
		rind *vs* seed	**<0.000**
		lint *vs* seed	**<0.000**
	young	rind *vs* lint	0.026
		rind *vs* seed	0.697
		lint *vs* seed	**0.009**
	rind	old *vs* young	0.199
	lint	old *vs* young	0.691
	seed	old *vs* young	**<0.000**

Table 2.S4. Univariate results for P and C content in field plants and the contrasts between variety and tissue. A Bonferroni correction was used for multiple comparisons (critical value P=0.0083). Bolded values indicate significance.

Variable	Source	df	F-ratio	P-value
P	variety	3	0.192	0.902
	tissue	3	53.92	**<0.000**
Individual contrasts				
terminal growth *vs.* true leaves				**<0.000**
terminal growth *vs.* squares				**<0.000**
terminal growth *vs.* bolls				**<0.000**
true leaves *vs.* squares				0.999
true leaves *vs.* bolls				**<0.000**
squares *vs.* bolls				**<0.000**
C	variety	3	6.424	**0.001**
	tissue	3	862.5	**<0.000**
Individual contrasts				
P203 *vs.* LA122				0.019
P203 *vs.* LA1203				**<0.000**
P203 *vs.* OL220				0.093
LA122 *vs.* LA1203				0.999
LA122 *vs.* OL220				0.999
LA1203 *vs.* OL220				0.426
Individual contrasts				
terminal growth *vs.* true leaves				**<0.000**
terminal growth *vs.* squares				**<0.000**
terminal growth *vs.* bolls				**<0.000**
true leaves *vs.* squares				**<0.000**
true leaves *vs.* bolls				**<0.000**
squares *vs.* bolls				**<0.000**

CHAPTER III

MACRONUTRIENT REGULATION IN A MAJOR PLANT PEST, *LYGUS HESPERUS* (HETEROPTERA: MIRIDAE)

Overview

The Western tarnished plant bug (WTPB), *Lygus hersperus*, is a highly polyphagous agricultural pest throughout North America. Feeding on over 330 plant species, this insect has the broadest host range ever documented for an insect. Despite the strong economic impacts this species has on crop productions throughout its range, very little is known about its nutritional ecology. Attempts to develop chemically-defined artificial rearing diets for this species have been tenuous, largely due to misunderstandings about its feeding mode and behavior. In this study, we performed choice and no-choice experiments to determine how *L. hesperus* regulates its intake of protein (p) and carbohydrates (c), two macronutrients that are tightly linked to survival and performance in insects. When allowed to select between two diets with different p:c ratios, we documented strong regulation for p and c, with final instar nymphs selecting for a slightly p-biased diet with a p:c ratio of 1.2:1. Our ability to detect the effects of diet p:c on performance when nymphs could not choose their diet was limited due to high mortality across artificial diets. Although survival was significantly higher on the high-p diet than the low-p diet, the overall cause of the observed mortality is not known. Progress in understanding the specific nutrient requirements of this economically important pest species has been slow, and these data offer insights into the regulation of

two physiologically important macronutrients. More work, however, is still needed to elucidate the complex feeding ecology of this species.

Introduction

Lygus hesperus, also known as the Western tarnished plant bug (WTPB), is a highly polyphagous mirid species found throughout North America. The WTPB has the broadest feeding niche reported for any arthropod (Young, 1986), and is documented to feed on over 330 host plants species, including 30 of the 70 angiosperms orders in existence in North America. This broad host range also makes the WTPB a significant economic pest on 21 of the 30 most important crops in the U.S. (Young, 1986). The WTPB, along with its close relative *Lygus lineolaris*, is becoming particularly problematic in cotton, specifically in the Southeastern U.S. (Layton, 2000; Esquivel and Mowery, 2007; Fournier et al., 2007). Its emergence as a cotton pest is primarily due to reductions in insecticide sprays used for boll weevil eradication (Armstrong and Camelo, 2003; Layton et al., 2003) and the increased use of transgenic *Bt*-crops, which do not control *Lygus* species, (Naranjo, 2005). Increased resistance to pyrethroids has also contributed to the increased pest status of *Lygus* (Snodgrass, 1996; Snodgrass and Scott, 2000). *Lygus* nymphs typically feed on wild hosts and move onto cotton as adults late in the season once wild hosts senesce (Snodgrass et al., 1984; Esquivel and Mowery, 2007). Adults damage crops by feeding on small squares (developing flowers) and bolls (fruits), which induces abscission (Greene et al., 1999; Layton, 2000; Zink and Rosenheim, 2005). This can result in reduced yields, delayed maturity, and vegetative

overgrowth (Greene et al., 1999; Layton, 2000, Rosenheim et al., 2006).

While much work has been done on characterizing the feeding ecology of chewing pests in agricultural systems, sucking pests have received less attention (Behmer, 2009). The WTPB are lacerate and flush feeders, meaning they have digestive enzymes in their saliva that allow them to liquefy plant tissues into a slurry (Agusti and Cohen, 2000; Backus et al., 2007; Celorio-Mancera et al., 2009; Esquivel, 2015), which is then ingested. Most of the nutritional research involving *Lygus* has focused on developing artificial rearing diets (Landes and Strong, 1965; Strong and Landes, 1965; Vanderzant, 1967; Raulston and Auclair, 1968; Strong, 1969; Debolt, 1982; Cohen, 2000a). Most of the diets developed for this species are oligidic or meridic, meaning they contain chemically undefined components such as wheat germ or chicken eggs. Of the chemically-defined diets that have been used to rear *Lygus*, all produced low survival and performance because they were originally designed for phloem- or xylem-feeding insects (Auclair and Raultson, 1966; Raulston and Auclair, 1968; Cohen, 2000b). Although *Lygus* species feed on plant tissues, their stylets can also reach the phloem and xylem (Strong, 1970; Backus et al., 2007; Esquivel, 2015). Despite this, failed attempts to rear *Lygus* on diet developed for phloem-feeders have shown that their nutritional preferences and requirements are very different from that of phloem-feeders (Cohen, 2000b). Overall, their specific nutritional requirements and their ability to contend with nutritional variability are still not well understood. As a result, there is a severe lack of information on the nutritional ecology of these pests, which undoubtedly limits our ability to develop effective control strategies.

Nutritional studies using a geometric framework have found that dietary macronutrients, particularly protein (p) and digestible carbohydrates (c), have important effects on insect survival and performance (Simpson and Raubehneimer, 1995; Behmer, 2009). Additionally, because insects can detect concentrations of amino acids and sugars in their food via chemosensory organs (Schoonhoven, 1968; Bernays and Chapman, 1994; Behmer, 2009), most insects actively regulate their intake of p and c to reach a specific ratio at which fitness is maximized (Raubenheimer and Simpson, 1999; Simpson and Raubenheimer, 1999; Behmer, 2009). This optimal p:c is termed an intake target (IT) (Raubenheimer and Simpson, 1999; Simpson and Raubenheimer, 1999; Behmer, 2009), and while ITs have been reported for several chewing insect species, especially grasshoppers (Raubenheimer and Simpson, 1993; Behmer et al., 2001; Behmer and Joern, 2008; Le Gall and Behmer, 2014) and caterpillars (Lee et al., 2002; Lee et al., 2004; Thompson and Redak, 2005; Despland and Noseworthy, 2006; Lee et al., 2006; Deans et al., *submitted*), the only ITs reported for sucking or fluid feedings insects are for the pea aphid (*Acyrthosiphon pisum*) (Abisgold et al., 1994; Simpson et al., 1995), honey bee (*Apis mellifera scutellata*) (Altaye et al., 2010), and fruit flies (*Drosophila melanogaster*) (Lee et al., 2008). The goal of this study was to determine the extent to which *L. hesperus* regulates its intake of protein and carbohydrates and also to document the effects that dietary p:c ratio have on survival and performance. Given that virtually all the insects examined to date have shown some degree of macronutrient regulation, we hypothesized that *L. hesperus* would show regulation for a specific IT and that dietary p:c would have significant impacts on their performance. Having a

documented IT for this species would be useful for optimizing artificial diets, which could improve rearing capabilities and enable more research to be done. Additionally, a better understanding of the nutritional requirements for this important pest species will also help to elucidate their feeding ecology in the field and may advance our ability to predict population distributions, movements, and dynamics in an attempt to improve control strategies and techniques.

Methods

Insects

Lygus hesperus egg packs were obtained from the Parajulee Lab at the Texas A&M AgriLife Research and Extension Center (Lubbock, TX). These eggs were used to start a colony, which was reared on a combination of snap peas (early instars) and artificial diet (Frontier Agricultural Sciences, Newark, DE). Nymphs from this colony were used in the first choice experiment. For the second experiment, which was conducted at a later date, we obtained egg packs from the USDA-ARS, Arid-Land Agricultural Research Center (Maricopa, AZ); hatching neonates from these eggs were in the second choice experiment and the no-choice experiment. Colonies and experimental individuals were maintained in an insect growth chamber (Model I-66VL; Percival Scientific, Perry, IA, USA) set at 25°C with a 14:10 L:D cycle.

Artificial Diet

The artificial diet of Debolt (1982) was modified to allow for the creation of diets with broad range of protein-to-carbohydrate ratios (p:c). This was done by eliminating the lima bean powder component and reducing the amount of wheat germ to 10% (dry mass), while adding vitamin-free casein and sucrose as a substitute for the macronutrient content contained in these ingredients. Other key ingredients were egg powder, lipids (cholesterol and linoleic acid), debolt salt mixture, and vitamins. All of the diets tested had a 74% total macronutrient content (by dry mass) (p+c). Food packs were made by sealing an aliquot of diet into 2.54 cm^2 Parafilm® packs. For early instars, the packs were stretched to thin out the Parafilm and facilitate feeding. In total, 5 different diets were made that had unique protein (p) and digestible carbohydrate (c) profiles. The original rearing diet from Debolt (1982) had a p:c ratio of p32:c42, and we created four other diets that corresponded to different cotton tissues (Deans et al., *in prep*), as cotton is a common host for *L. hesperus* throughout the Southern U.S. The four other diets had p:c ratios of p24:c50 (bolls), p37:c37 (leaves), p50:c24 (squares), and p60:c14 (squares), which corresponded to the range of ratios found in different cotton tissues.

Choice Experiment

Choice experiments were performed to determine the extent to which *L. hesperus* nymphs regulate their intake of p and c. This was done by measuring total p and c consumption for nymphs offered the choice between two foods that differed in p:c ratio. The ability to maintain a specific balance of p and c intake (p:c ratio) across different

diet pairings indicates that nymphs are actively regulating for macronutrients and selectively feeding to achieve a specific intake target (IT), or p:c ratio. Two choice experiments were conducted. The first tested three diet pairings (see Fig. 3.1a): (1) p24:c50 vs p37:c37, (2) p24:c50 vs p50:c24 and (3) p32:c42 vs p50:c24. These diets covered a range of p:c ratios from 0.48-2.0. This first experiment indicated that the intake target (IT) for *L.hesperus* was P-biased, so a second choice experiment was conducted to test a diet pairing that encompassed a more P-biased nutritional space. Here, a p37:c37 diet was paired with a p60:c14 diet (see Fig. 3.1b). In both experiments, newly hatched nymphs were reared on snap peas until molting to the 4th instar (final), weighed, and then transferred to individual 8 oz. clear plastic containers with wire mesh lids. Individuals were then assigned to a treatment,

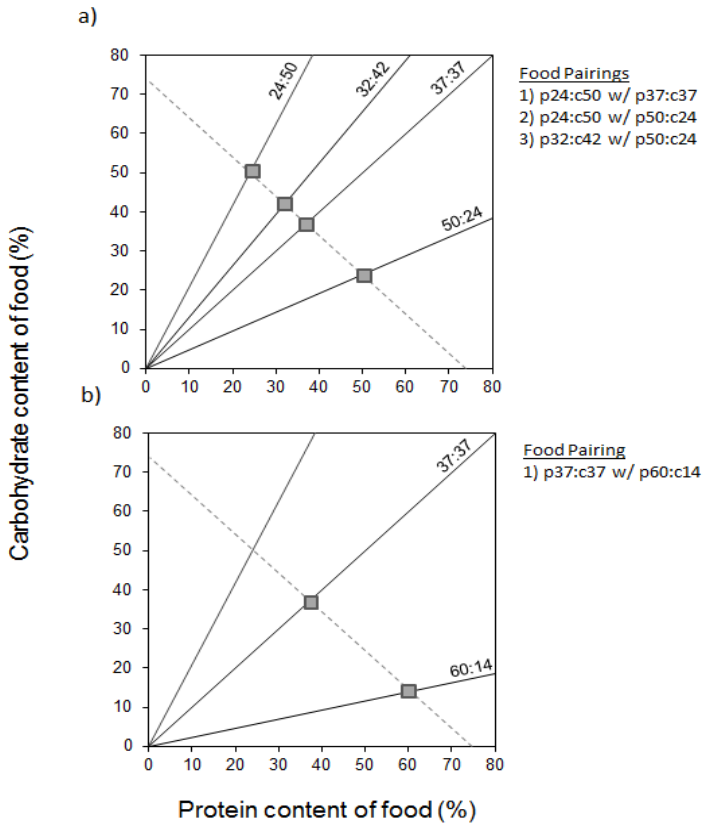

Figure 3.1. The carbohydrate and protein content of the different diets tested in the first choice experiment (a) and the second choice experiment (b). All diets were 74% macronutrients by dry mass and each line indicates the p:c ratio of each diet.

each consisting of a unique food pairing. Food packs were placed on top of the mesh lids, as in Fig. 3.2. In the first experiment, there were 20 replicates per treatment, and in the second experiment there were 8 replicates per treatment. All nymphs were housed in a growth chamber at 25°C on a 14:10 L:D photoperiod.

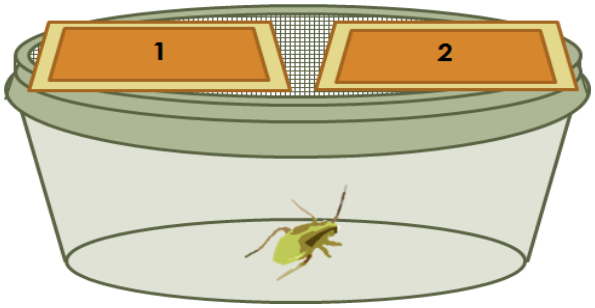

Figure 3.2. A drawing of the experimental set-up for the choice experiments. Nymphs were housed individually in a clear 8 oz. plastic container with a mesh lid, and each food pack was placed on the outer surface of the lids. Nymphs were able to then feed through the mesh on each food pack.

No-Choice Experiment

Newly hatched neonates were reared on snap peas until the 3rd instar, weighed, and then transferred to individual containers (1 oz. clear cups with paper lids). Each individual was assigned to one of three diet treatments, each consisting of a different p:c ratio. Treatments diets had the following p:c ratios: (1) p24:c50, (2) p37:c37, and (3) p50:c24 (Fig. 3.3), and were selected to encompass the range of p:c ratios found in different cotton tissues (Deans et al., *in prep*), which *L. hesperus* commonly feed on. Food packs were placed inside each container. Once nymphs molted into adults they were weighed. Survival, mass gain, and developmental time were recorded, as well as food consumption. Each treatment contained 30 replicates housed in a growth chamber at 25°C on a 14:10 L:D photoperiod.

Data Analysis

For the choice experiments, differences in food pack consumption were used to test for non-random feeding using a one-sample t-test. A MANCOVA (with initial mass as a covariate) was used to test for differences in p and c consumption across treatments. A non-parametric Kruskal-Wallis test was used to determine if there were differences in the p:c ratio of consumed food across treatments due to difficulties meeting normality and equal variance assumptions. A Mann Whitney-U test was used for post hoc comparisons, with a strict Bonferroni correction to account for multiple comparisons. Differences in survival and developmental time across treatments were determined using a Kaplan-Meier survival analysis, and differences in mass gain across treatments were analyzed using an ANOVA.

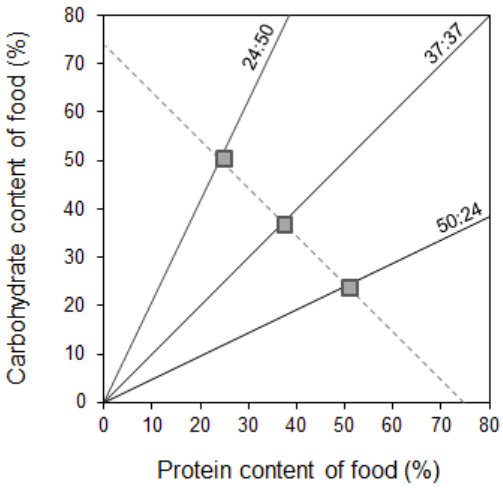

Figure 3.3. The carbohydrate and protein content of the different diets tested in the no-choice experiment. All diets were 74% macronutrients by dry mass and each line indicates the p:c ratio of each diet. The food p:c ratios tested were p24:c50, p37:c37, and p50:c24.

Results

Choice Experiments

Experiment 1

Nymphs in the first choice experiment exhibited non-random feeding on each treatment (Fig. 3.4), indicating that they were showing a preference for one of the food packs. The MANCOVA results in Table 3.1 show that differences in feeding resulted in significant differences in p and c ingestion between treatments; however, the univariate results indicate that these differences were due to variability in p consumption only (Fig. 3.5a). Protein consumption was significantly lower in the p24:c50 vs p37:c37 treatment than the other two treatments, while c consumption did not differ between treatments. Similarities in p and c consumption across food pairings can indicate that nymphs are actively regulating their macronutrient intake to reach a specific p:c ratio. The MANCOVA results showed that the p24:c50 vs p37:c37 treatment was significantly different from the p24:c50 vs p50:c24 and the p32:c42 vs p50:c24 treatments; however, the p24:c50 vs p50:c24 and

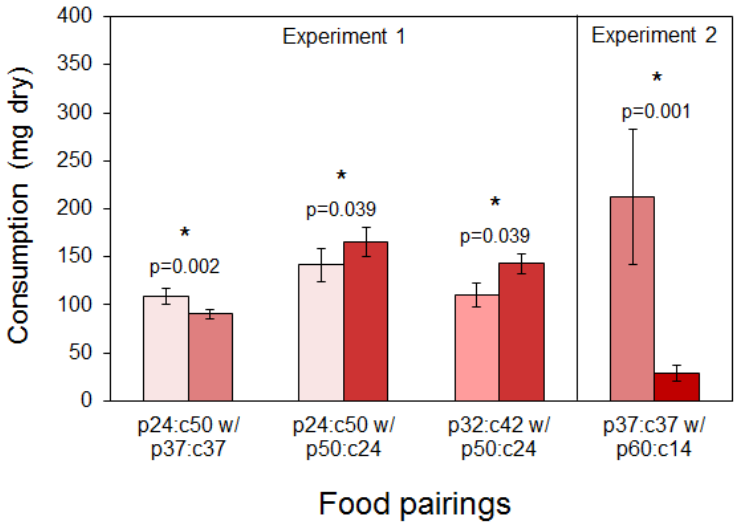

Figure 3.4. Consumption across both food packs for both choice experiments. Asterisks indicate that consumption across food packs was significantly different according to a one-sample t-test. The bar of the left corresponds to the first diet listed on the x-axis, while the bar to the right corresponds to the second diet listed.

Table 3.1. MANCOVA and univariate results for p and c consumption across treatments in the first choice experiment. Individual contrasts have a strict Bonferroni adjusted p-value of 0.0167. Bolded values indicate significance at the α=0.05 level.

	Variable	Source	Hypothesis df	Error df	F Ratio	P-value
MANCOVA	p and c	treatment	4	52	13.82	**<0.000**
		initial mass	2	25	0.873	0.430
	Individual Contrasts					
	[p32:c42 w/ p50:c24] vs [p24:c50 w/ p50:c24]		2	19	15.25	**<0.000**
	[p32:c42 w/ p50:c24] vs [p24:c50 w/ p37:c37]		2	14	80.81	**<0.000**
	[p24:c50 w/ p50:c24] vs [p24:c50 w/ p37:c37]		2	15	59.35	**<0.000**
Univariate	p consumption	treatment	2		9.31	0.001
	Pairwise Comparisons					
	[p32:c42 w/ p50:c24] vs [p24:c50 w/ p50:c24]					1.000
	[p32:c42 w/ p50:c24] vs [p24:c50 w/ p37:c37]					**0.004**
	[p24:c50 w/ p50:c24] vs [p24:c50 w/ p37:c37]					**0.001**
	c consumption	treatment	2		3.07	0.064

Table 3.2. Kruskal-Wallis and Mann Whitney-U (pairwise comparisons) results for the effect of treatment on the p:c ratio of consumed food in first choice experiment. Bolded values and different letters indicate significant differences at the α=0.05 level.

Variable	Source	Hypothesis df	X^2	P-value	P:C Ratio	
p:c ratio	treatment	2	9.31	**0.001**		
Pairwise Comparisons						
[p32:c42 w/ p50:c24] vs [p24:c50 w/ p50:c24]					0.680 ± 0.01	A
[p32:c42 w/ p50:c24] vs [p24:c50 w/ p37:c37]					1.27 ± 0.045	B
[p24:c50 w/ p50:c24] vs [p24:c50 w/ p37:c37]					1.10 ± 0.040	B

Table 3.3. Kaplan-Meier analysis for survival and developmental time in the no-choice experiment.

Variable	Source	Hypothesis df	X^2	P-value
survival	treatment	2	7.53	**0.023**
developmental time	treatment	2	1.17	0.558
Pairwise Comparisons (survival)				
p24:c50 vs p37:c37			2.84	0.092
p24:c50 vs p50:c24			7.13	**0.008**
p37:c37 vs p50:c24			1.10	0.294

the p32:c42 vs p50:c24 treatments showed the same p and c consumption (Table 3.1). The p:c ratio of consumed food was also the same for these treatments (Table 3.2). The results show strong p and c regulation across the p24:c50 vs p50:c24 and the p32:c42 vs p50:c24 treatments. Because the average c consumption did not vary significantly across pairings and p consumption was the same across the p24:c50 w/ p50:c24 and p32:c42 w/ p50:c24 treatment, we can average the c and p consumption across similar treatments to calculate an approximate IT. Average c consumption across all treatments was 94.23 mg and average p consumption across the p24:c50 w/ p50:c24 and p32:c42 w/ p50:c24 treatments was 112.00 mg, giving us a slightly p-biased IT of 1.20. There were no

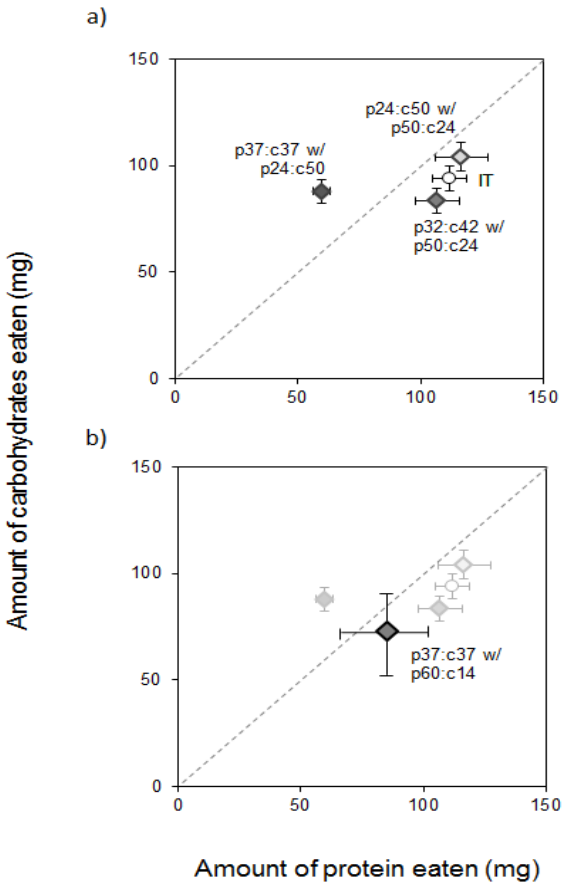

Figure 3.5. Protein and carbohydrate consumption across choice experiment 1 (a) and choice experiment 2 (b). The intake targets (IT) for experiment 1 was calculated by averaging p consumption across the p24:c50 w/ p50:c24 and p32:c42 w/ p50:c24 pairings and c consumption across all pairings. The dotted line indicates a 1:1 p:c ratio for reference. Error bars show ± 1SE.

differences in survival, developmental time (X^2=1.23, df=, p=0.542), or mass gain ($F_{2, 26}$=2.62, p=0.092) across treatments.

Experiment 2

Nymphs in the second choice experiment showed non-random feeding in the (Fig. 3.4), with significantly higher consumption on the p37:c37 diet. Average p consumption was 84.82 mg (± 17.9) and average c consumption was 71.78 mg (± 18.51) (Fig. 3.5b). The overall p:c ratio of consumed food was 1.11 (± 0.05), which was similar to that found in the first choice experiment. Mortality was very low, with only one death recorded. Average mass gain was 1.7 mg and was similar to that in the first experiment.

No-Choice Experiment

Although mortality was very high across all treatments, the Kaplan-Meier analysis showed significant differences in survival across food treatments (Table 3.3). The p24:c50 had the highest mortality, with only one individual out of 30 surviving to adulthood (96% mortality), and the p50:c24 treatment had significantly higher survival with 63% mortality. The p37:c37 treatment was intermediate with six survivors (80% mortality). The Figure 3.6 shows the survival curves for each treatment. For those that survived, there were no differences in sex ratio (X^2=1.52, df=2, p=0.467), developmental time (Table 3.3), or mass gain ($F_{2, 15}$=2.49, p=0.117).

Discussion

We found that *L. hesperus* actively regulates for p and c, selecting for a slightly p-biased IT of 1.2. In the first choice experiment, we only observed regulation for p across two of the three food pairings, suggesting that individuals in the p24:c50 w/ p37:c37 treatment, which represented the most c-biased portion of nutritional space tested, could not obtain their requirements for p on the available diets. In the second choice experiment, we found that nymphs selected for a p:c ratio of 1.1:1, which was very similar to the IT calculated in the first choice experiment. This p-based IT makes sense considering that *Lygus* species typically feed on p-rich fruiting structures (Snodgrass et al., 1984; Greene et al., 1999; Layton, 2000; Zink and Rosehneim, 2005).

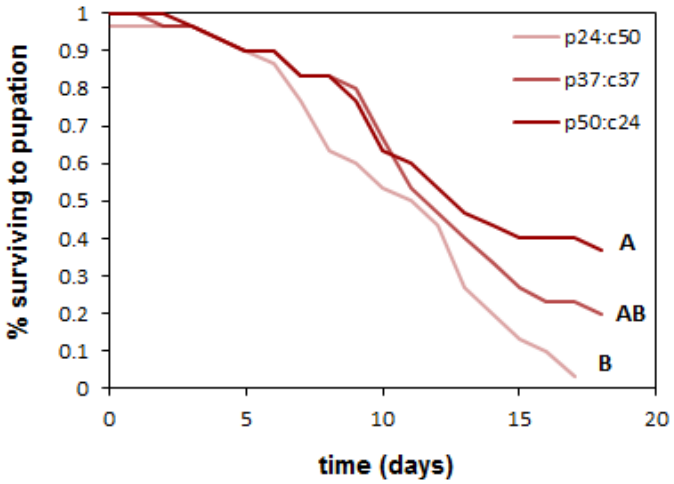

Figure 3.6. Survival curves for each food treatment in the no-choice experiment. Line endpoints indicate the time it took for all replicates to reach adulthood.

The results of our no-choice study did not show any differences in performance across food p:c; however, our ability to detect differences in developmental time and mass gain was severely impacted by the high mortality observed across all our diet treatments. It is clear that the nymphs were feeding on the food packs, as mortality didn't significantly increase until after 5-7 days, but it is unclear as to what caused the sharp decline in survival. Across both artificial diets and other plant resources, such as green

beans which are commonly used to rear *Lygus*, mortality is highest in the early to mid nymphal instars (Strong and Kruitwagen, 1968; more). It is possible that the dietary requirements are more rigid for early instar nymphs than later instars and adults. Although we had high mortality in our no-choice study, mortality in our choice experiments, which were performed with final instar nymphs, was very low. Also, surviving adults from our choice studies had longevity that was comparable to those in our rearing colonies, further suggesting that the observed mortality may be related to using earlier instar nymphs in the no-choice experiment.

Much work has been done on developing artificial diets for *Lygus* (Landes and Strong, 1965; Strong and Landes, 1965; Vanderzant, 1967; Raulston and Auclair, 1968; Strong, 1969; Debolt, 1982; Cohen, 2000a), and although both the Debolt (1982) and the Cohen (2000) diets have been successfully used to maintain colonies with reproducibly high performance, the main components of these diet are chemically undefined. Earlier work by Auclair and Raulston (1966), Raultson and Auclair (1968), Landes and Strong (1965), and Vanderzant (1967) that attempted to rear *Lygus* on chemically-defined diets developed for aphids proved unsuccessful. This was likely due to differences in feeding modes between *Lygus*, which feed on semi-solid material, and aphids, which feed only on fluids. In fact, Cohen (2000b) reported that attempts to filter out the solid materials found in the Debolt (1982) diet (i.e. wheat germ, lima bean meal, etc.) resulted in reduced performance and indicated that particulates are an important element in artificial diets for *Lygus*. This requirement for semi-solid dietary components has made the development of chemically-defined diets, which typical contain solutes like casein and

sucrose, difficult. The diet we used in the no-choice experiment did contain a small amount of wheat germ (10% by dry mass), but it is possible that concentration was not high enough to support optimal nymphal growth.

All the diets contained the same percentage of lipids, vitamins and minerals, only varying in p and c content. If the mortality was due to some sort of lipid or micronutrient deficiency, one would expect mortality to be equal across diets. It is, however, possible that the treatments had an impact on feeding behavior. Strong and Kruitwagen (1970) showed that across concentrations of dietary sucrose, amino acids, lipids, vitamins, and salts the only component that stimulated *Lygus* feeding was amino acids. In light of this, it is possible that p50:c24 diet stimulated more feeding than the others due to its high proportion of p which resulted in lower mortality. In this case, low mortality may have resulted from low consumption due to the nymph's inability to recognize the low-p diet as an acceptable resource. Despite this, mortality was still relatively high in the p50:c24 treatment compared to that reported on the meridic artificial diets (Debolt, 1982; Cohen, 2000a).

Perhaps the most likely explanation may be that an outside and unanticipated stressor was present and affecting the nymphs. Although antibiotics were added to the diets, we did observe some microbial growth in the diet packs over time. These packs were replaced with new food packs when observed, but it is possible that this microbial growth was present and impacting the nymphs before it was visible to us. Diet has been shown to impact susceptibility to both toxins and pathogens. In caterpillars, infected individuals showed significantly better survival and performance on high-p diets (Lee et

al., 2006; Lee et al., 2008). If an infectious agent was responsible in some way for the observed mortality, it could explain why nymphs reared on the p50:c24 diet had significantly higher survival than the p24:c50 diet. Other studies have shown that insects are more effective at dealing with toxins when they are reared on a diet that matches their IT (Simpson and Raubenheimer, 2001; Shikano and Cory, 2014; Orpet et al., 2015). The p37:c37 diet was the closest of the diets tested to the IT found for *L. hesperus*. Since there was no significant difference between survival on this diet and that of p50:c24, it is also possible that a toxin, perhaps one produced by a mold or fungus, could have caused the diet-specific trends in survival in the no-choice experiment.

In conclusion, understanding the nutritional ecology of *L. hesperus* has proved to be more complicated than anticipated. We detected strong regulation for p and c, with the IT showing a slightly p-biased preference of diets with a 1.1-1.2 p:c ratio. However, due to problems with high mortality in early instars, we had a difficult time determining the effects of diet p:c on performance. The rather unique feeding mode of *Lygus* species has made the development of successful chemically-defined rearing diets difficult, further hindering our understanding of their specific nutritional requirements. This study provides data on requirements for p and c, which are two macronutrients that have been shown to have significant effects on insect survival and performance. These data will be useful for improving rearing diets and will hopefully spur future research on the nutritional ecology of this economically important species.

Acknowledgements

We would like to thank Owen McSpadden for helping us learn the proper rearing techniques and for supplying us with eggs. We would also like to thank James Hagler for supplying us with eggs for the second round of our experiments. Thank to Jesus Esquivel for assisting in field collections and providing us with general information about Texas host plants. This project was funded by the C. Everette Sayler Fellowship and the Charles Parencia Endowment through Texas A&M University.

CHAPTER IV

REVISITING MACRONUTRIENT REGULATION IN THE POLYPHAGOUS HERBIVORE *HELICOVERPA ZEA* (LEPIDOPTERA: NOCTUIDAE): NEW INSIGHTS VIA A GEOMETRIC APPROACH

Overview

Insect herbivores that ingest protein and carbohydrates in physiologically-optimal proportions and concentrations show superior performance and fitness. The first-ever study of protein-carbohydrate regulation in an insect herbivore was performed using the polyphagous agricultural pest *Helicoverpa zea*. In that study, experimental final instar caterpillars were presented two diets – one containing protein but no carbohydrates, the other containing carbohydrates but no protein – and allowed to self-select their protein-carbohydrate intake. The results showed that *H. zea* selected a diet with a protein-to-carbohydrate (p:c) ratio of 4:1. At about this same time, the geometric framework (GF) for the study of nutrition was introduced. The GF is now established as the most rigorous means to study nutrient regulation (in any animal). It has been used to study protein-carbohydrate regulation in several lepidopteran species, with results producing a range of self-selected p:c ratios between 0.8-1.5. Given the economic importance of *H. zea,* and it's extremely protein-biased p:c ratio of 4:1 relative to those reported for other lepidopterans, we decided to revisit its protein-carbohydrate regulation. Our results, using the experimental approach of the GF, show that *H. zea* larvae self-select a p:c ratio of 1.6:1. This p:c ratio strongly matches that of its close

relative, *Heliothis virescens,* and is more consistent with self-selected p:c ratios reported for other lepidopterans. Having accurate protein and carbohydrate regulation information for an insect herbivore pest such as *H. zea* is valuable for two reasons. First, it can be used to better understand feeding patterns in the field, which might lead to enhanced management. Second, it will allow researchers to develop rearing diets that more accurately reflect larval nutritional needs, which has important implications for resistance bioassays and other measures of physiological stress.

Introduction

The ability of insect herbivores to acquire an optimal mixture of dietary nutrients has profound effects on their performance and fitness (Bernays and Bright, 1993; Bernays and Minkenberg, 1997; Raubenheimer and Jones, 2006; Unsicker et al., 2008; Behmer, 2009). In general, plant nutrient content is highly variable, both spatially and temporally (Elser et al., 2000; McGroddy, Daufresne, and Hedin, 2003; Deans, 2014), indicating that the majority of herbivores forage in a highly heterogeneous nutritional landscape. To deal with this variability, insect herbivores assess the nutrients present in different plant tissues and regulate the intake of specific nutrients to meet their physiological demands (Raubenheimer and Simpson, 1999; Simpson and Raubenheimer, 1999; Behmer, 2009). The process of acquiring the optimal balance of key nutrients to fuel growth and reproduction strongly impacts insect performance, with consequences for the evolution of plant-insect interactions and host-plant associations (Bernays and Chapman, 1994; Bernays and Bright, 2005), dispersal and movement patterns (Simpson

et al., 2006; Bazazi et al., 2008; Srygley et al., 2009; Simpson et al., 2010; Hansen et al., 2011) and even the evolution of higher order social interactions (Guttal et al., 2012; Lihoreau et al., 2014, 2015). For these reasons, delineating the nutritional requirements of an insect species is integral to understanding its feeding ecology, life history strategies, and physiological capabilities.

The cotton bollworm, *Helicoverpa zea*, is a highly polyphagous agricultural crop pest that feeds on over 100 different host plants in North America (Fitt, 1989). *H. zea* was also the first species to be used for exploring nutrient regulation in herbivorous insects (Waldbauer et al., 1984). In this study, a choice test was performed to determine the extent to which *H. zea* larvae regulated their protein (p) and carbohydrates (c) intake. To do this, larvae were offered one of two artificial diet pairings over the course of their final instar, either a diet with a protein-to-carbohydrate ratio (p:c) of 100:0 (all protein) and one with a p:c of 0:100 (all carbohydrates), or two diets both with a 50:50 ratio. The consumption results indicated that, when allowed to self-select, larvae ingested a diet with an average p:c ratio of 80:20, or 4:1; in GF parlance, this self-selected p:c ratio is referred to as an intake target (IT) (Simpson and Raubenheimer, 1995).

Since Waldbauer et al. (1984), protein and carbohydrate regulation has been tested in several lepidopteran species using the experimental approach of the geometric framework (GF), including *Heliothis virescens* (Lee et al., 2006), *H. subuflexa* (Lee et al., 2006), *Manduca sexta* (Thompson and Redak, 2005), *Malacasoma disseria* (Despland and Noseworthy, 2006), *Spodoptera exigua* (Merkx-Jacques et al., 2008), *S. exempta* (Lee et al., 2004), and *S. littoralis* (Lee et al., 2004). Across these species, the

ITs range from slightly carbohydrate-biased ratio for *S. exempta* (0.8:1) to slightly protein-biased ratio for *H. virescens* (1.5:1) and *S. littoralis* (1.3:1), with several species selecting for a balanced 1:1 p:c ratio. Comparatively, the 4:1 IT for *H. zea* as determined in Waldbauer et al. (1984) stands apart from these other caterpillar species because it is extremely protein-biased. In particular, it is much more protein-biased than the 1.5:1 IT found for *H. virescens* (Lee et al., 2006), a close relative to *H. zea*.

Given the economic significance of *H. zea* and the major discrepancy between Waldbauer et al. (1984) and other lepidopteran studies employing the GF, we wanted to reassess protein-carbohydrate regulation in *H. zea*. We had two objectives. The first was to determine the IT for *H. zea* using the experimental approach of the GF. To do this, a choice-experiment was performed in which individuals were offered pairings of two diets that differed in their p:c ratios; for each treatment newly-molted final instar caterpillars were maintained individually and consumption of each food was measured over the final instar. The total amount of protein and carbohydrates consumed over the study was then used to calculate the IT. The second objective was to understand how diet p:c impacts performance when larvae cannot choose. This was done with a no-choice experiment by rearing larvae from neonate to pupation on diets with a specific p:c ratio and then measuring growth rate, developmental time, and pupal mass. Given the IT results for other caterpillar species, particularly *H. virescens*, we expected the IT to be only slightly protein-biased, approximating the upper range found in these other studies. We also hypothesized that performance in the no-choice study would be best on the diet treatment that most closely matched the IT calculated from the choice experiments,

given that ITs have been shown to be functionally optimal (Behmer and Joern, 2008; Roeder and Behmer, 2014).

Methods

Insects

H. zea eggs were purchased from Benzon Research (Carlisle, PA). Upon hatching, neonates were individually placed, using a fine-tipped paint brush, into 1 oz. clear condiment cups with paper lids. Each cup also contained one or two blocks of experimental food that differed in soluble protein and digestible carbohydrate content (see below). All individuals were kept in a growth chamber (Model I-66VL; Percival Scientific, Perry, IA, USA) set at 25°C with a 14:10 L:D cycle for the duration of each experiment.

Artificial Diet

The synthetic diet used in this study was originally developed by Ritter and Nes (1981), and then later modified as described by Jing et al. (2013). The key ingredients were vitamin-free casein, sucrose, cellulose, Wesson's salt mix, Torula yeast, lipids (cholesterol, linoleic and linolenic acid) and vitamins. In total, 11 different diets were made that had unique protein and digestible carbohydrate profiles. All other ingredients, except for cellulose, were held constant between the different diets; the amount of cellulose in a diet varied inversely with total macronutrient content. The original diet

from Ritter and Nes (1981) contained 34% protein (p) and 12% sucrose (digestible carbohydrate (c)). This diet (p34:c12), plus three others (p12:c34, p17:c29 and p23:c23) had the same total macronutrient content (p+c) of 46%, but varied in p:c ratio from 0.35 to 2.8 (see Fig. 4.1a). Collectively, these four diets were used in various combinations in a choice experiment (described below).

The remaining seven diets (see Fig. 4.1b) were used in a no-choice experiment (see below). Three of these diets had total macronutrient content of 21%, but varied in p:c ratio from 0.4 to 2.5 (p6:c15, p12:c9 and p15:c6). The next three had a higher total macronutrient content of 42% with the same p:c ratios (p12:c30, p24:c18 and p30:c12), and the final diet had total macronutrient content of 68% and a p:c ratio of 1.6 (p42:c26). This resulted in the same three ratios being tested at two different total macronutrient concentrations: 0.4 (6p:15c and 12p:30c), 1.3 (12p:9c and 24p:18c), and 2.5 (15p:6c and 30p:12c) (see Fig. 4.1b). To maintain ecological relevance to natural conditions, these ratios and concentrations were selected to mimic the empirically-determined range of macronutrient content found in different cotton tissues under different growing conditions (Deans, 2014). Cotton is a common resource for *H. zea*, and as a result, larvae are likely to encounter resources of this quality in a natural setting. Table 4.1 shows the relationship between our experimental diets and the nutrient values for different cotton tissues.

All of the experimental diets were mixed as dry ingredients with a slightly warm 1% agar solution. After cooling, the diets were cut into blocks and presented to the experimental caterpillars. In this way caterpillars received both nutrients and water.

Experimental Protocol

Choice Experiment

All caterpillars were reared on the original Ritter and Nes (1981) diet (p34:c12, 46% total macronutrients) from hatching through to the start of the final instar. Upon molting to the final instar, larvae were weighed, then transferred to a petri-dish (with holes in the lid for ventilation) and offered two foods that differed in p:c ratio. There were three unique treatments: (1) p12:c34 paired with p34:c12, (2) p17:c29 paired with p34:c12 and (3) p23:c23 paired with p34:c12. Diet cubes were individually weighed and placed at opposites ends of the petri-dish (100 mm diameter). Both diet cubes were replaced every 1-2 days so that both diets were always available to the larvae. Consumption of each diet was measured by obtaining the wet and dry mass of each diet portion, using a wet-dry mass regression calculated separately to determine differences in initial and final dry mass. The total amount of protein and carbohydrates consumed was calculated using the total amount eaten from each block of food. Survival to pupation, developmental time until pupation, and pupal mass were measured for each experimental insect. There were 15 replicates per treatment and the sex ratio for each treatment, identified at the pupal stage, was a 1:1 ratio.

No-Choice Experiment

Larvae were reared on one of the seven experimental diets from neonate through to pupation. Individual food blocks were replenished as needed, but changed a minimum of every 2-3 days to prevent the food from drying out. Developmental time until

pupation, pupal mass, and growth rate were measured for each experimental insect. There were 15 replicates per treatment and the sex ratio for each treatment, identified at the pupal stage, was a 1:1 ratio. Survival across all treatments was high, with only one death occurring in the p42:c26 treatment.

Table 4.1. The empirically-determined range of total macronutrient content (%) and p:c ratios for different cotton tissues across both field and greenhouse conditions (only greenhouse values are reported for seed) (Deans, 2014).

Tissue	Concentration (%)	P:C Ratio
Terminal growth	34.0-41.8	1.18-1.19
True leaves	26.9-41.8	1.16-1.68
Squares	17.7-30.6	1.18-14.80
Bolls	29.5-41.3	0.41-1.17
Seed	68.0	1.60

Data Analysis

For the choice experiment, protein-carbohydrate consumption points were analyzed using MANCOVA techniques (with initial mass at the final instar used as a covariate). For both experiments an ANCOVA was used to determine differences in pupal mass and growth rate, and a Kaplan-Meier survival analysis (specifically the Mantel Cox test) was used to determine differences in developmental time between treatments. The Tukey-b test was used for ANCOVA post-hoc tests. All analyses were done using SPSS version 21 for Windows (SPSS Inc., Chicago, IL, USA).

Results

Choice Experiment

Strong regulation for P and C was apparent in the choice experiment. The MANCOVA results showed that P and C consumption ($F_{4,80}$=1.44, P=0.229) were statistically similar across all treatments, indicating that larvae standardized their consumption of these two macronutrients across all diet pairings (Figure 4.2). Univariate tests also showed that this was a result of both total protein consumption (F_2=2.60, P=0.087) and carbohydrate consumption (F_2=0.57, P=0.57) being similar across all three treatments (Table 4.2). Average protein consumption throughout the experiment was 103.0 mg (± 6.7), and average carbohydrate consumption was 76.3 mg (± 4.4). This resulted in a protein-biased intake target of 1.6 (± 0.14). There was no mortality during this experiment and Figure 4.3 shows that there was also no effect of diet treatment on

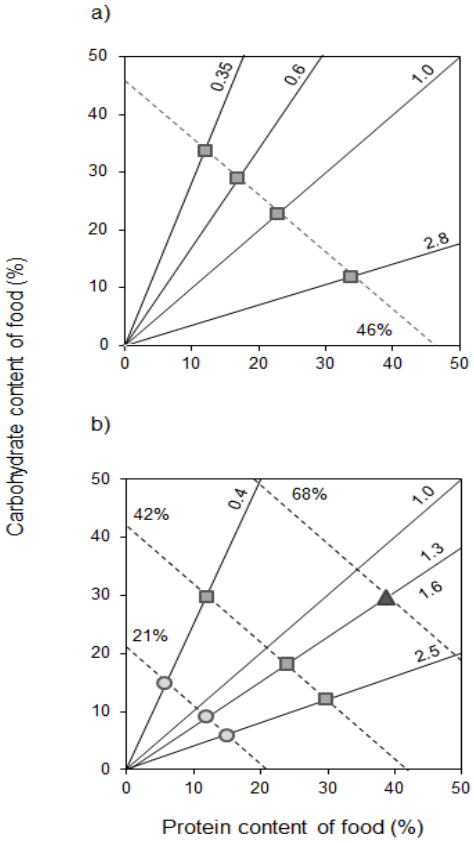

Figure 4. 1. The protein-carbohydrate content of the experimental foods for the (a) choice and (b) no-choice experiments. For each panel, individual points indicate the protein (x-axis) and carbohydrate (y-axis) content of the test food, expressed as percent dry mass of the food. Each panel also has 4 rail, that represent the different food p:c ratios (indicated at the end of each rail). Dashed lines that intersect the rails show total caloric content of individual diets.

developmental time (Mantel-Cox, X^2=1.50, df=2, P=0.472) or pupal mass (F_2=0.551, P=0.581).

Table 4.2. Results of the MANCOVA (with initial mass as a covariate) for consumption across treatments in the choice experiment.

Variable	Factor	df	F-ratio	P-value
P+C consumption	Treatment	4	1.44	0.229
	Initial mass	2	3.85	0.030

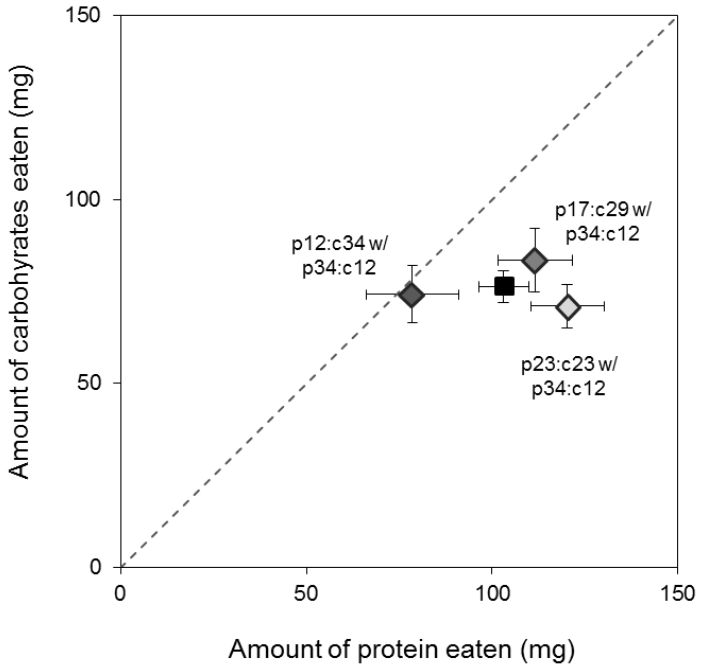

Figure 4. 2. Self-selected protein-carbohydrate intake from the choice experiment. Each diamond shows the mean (±SEM) amount of protein and carbohydrate eaten for each of the three choice treatments; they were not statistically different from one another. The black square shows the mean (±SEM) amount of protein and carbohydrate eaten when calculated across all treatments.

No-Choice Experiment

Developmental Time

There was a significant effect of diet on developmental time (Mantel-Cox, X^2=37.64, df=6, P<0.0001), and an influence of both total macronutrient concentration and p:c ratio was apparent. Larvae on the 6p:15c diet, the most carbohydrate-biased at 21% total nutrients, exhibited the longest developmental time, while those on the same ratio at 42% total nutrients took significantly less time to pupate. Also, across both the 21% and 42% diets, larvae on the protein-biased ratios (12p:9c, 15p:6c, 24p:18, 30p:12c) had consistently shorter developmental times (Figure 4.4a).

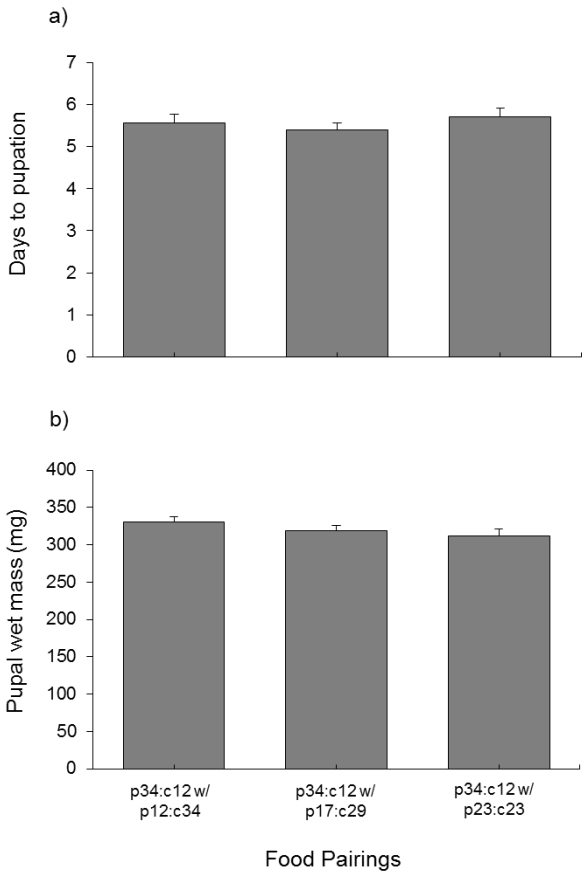

Figure 4. 3. Performance for caterpillars from the choice experiment. Panel (a) shows mean (±SEM) days to pupation, and panel (b) shows mean (±SEM) pupal wet mass. The x-axis indicates the diet pairings for each treatment.

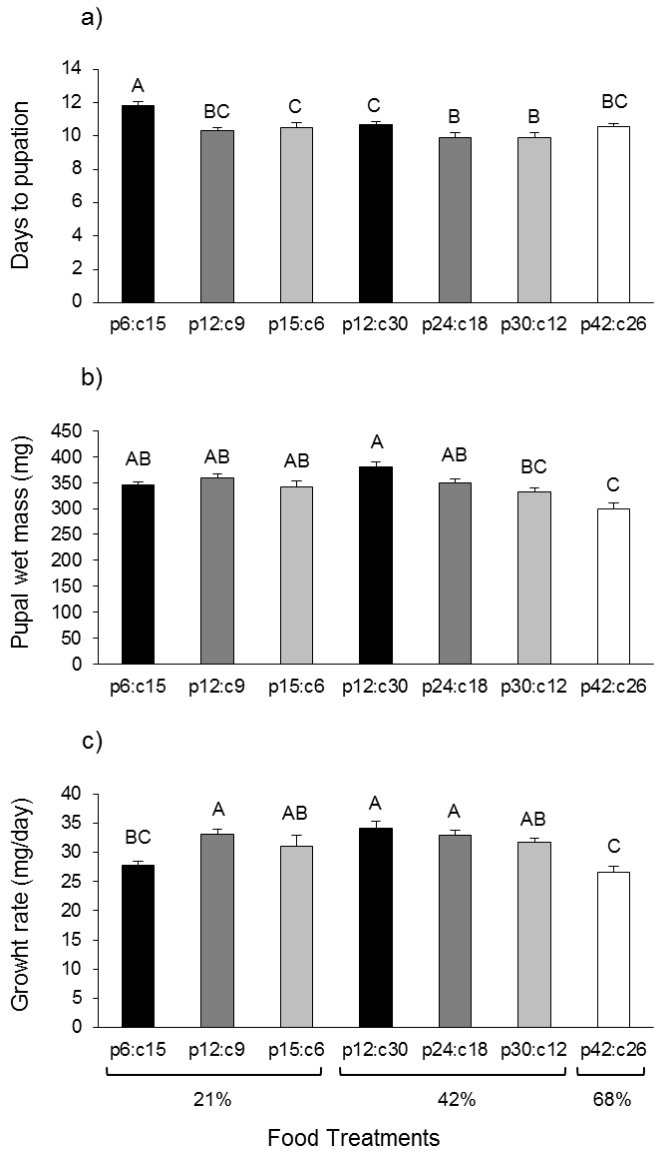

Figure 4. 4. Performance in the no-choice experiment. Panel (a) shows mean (±SEM) days to pupation, panel (b) shows mean (±SEM) pupal wet mass, and panel (c) shows mean (±SEM) growth rate (mg/day). Foods are grouped by their total macronutrient content, and then by increasing p:c ratio. Bars of similar color have similar p:c ratios. Different letters above each bar indicate significant differences ($P < 0.05$).

Pupal Mass

There was a significant diet effect on pupal mass ($F_{6,114}$=6.411, P<0.0001). Among the 21% diets, p:c ratio had no impact; however, at 42% total nutrients, the most carbohydrate-biased diet (12p:30c) showed significantly higher pupal mass (Figure .4b). In general, pupal mass was lower on the high-protein diets, while mass was relatively stable over carbohydrate concentrations.

Growth Rate

There was a significant effect of diet treatment on growth rate ($F_{6,100}$=6.78, P<0.0001). Figure 4.4c shows that larvae on the high 68% nutrient diet (42p:26c) had the lowest growth rate overall, and for those on the dilute diets (21% total nutrients), the moderate p:c ratio (12p:9c) produced the highest growth rate. There was no significant difference in growth rate across the different p:c ratios for the 42% total nutrient diets.

Discussion

In sharp contrast to the earlier work of Waldbauer et al. (1984), we found that *H. zea* larva select dietary protein and carbohydrates in a 1.6:1 ratio, rather than a 4:1 ratio. This result is much more consistent with the ITs of other lepidopterans that have been measured in GF studies and most closely matches that found for *H. virescens* (Lee et al., 2006; Roeder and Behmer, 2014), a generalist New World species that is closely related to *H. zea*. These results support our initial hypothesis that the IT for *H. zea* would be slightly protein-biased and have a similar IT to *H. virescens*.

So why did we find such a different IT compared to Waldbauer et al. (1984)? Important methodological differences likely provide the best explanation. In our study, we had three unique diet pairings (Figure 4.2), and the p:c ratios of the four foods we used (Figure 4.1) mimicked concentrations found in cotton (Table 4.1) (Deans, 2014), a natural host plant on which *H. zea* commonly feeds. In contrast, Waldbauer et al. (1984) only used two diet pairings, generated from three different diets. The first pairing was a control treatment were both diet cubes were identical – each had a 50:50 p:c ratio. The second treatment paired a p100:c0 diet with a p0:c100 diet. However, the use of diets that contain only protein or carbohydrates does not accurately represent a resource that an herbivore would encounter in nature, and the use of such extreme diets may have resulted in aberrant feeding behavior. The strong preference caterpillars showed for the protein containing diets (Waldbauer et al. 1984) may simply reflect the possibility that the carbohydrate-only diet was barely recognizable as food. Interestingly, Mormon crickets in the field show very little interest in carbohydrate-only artificial diets (Simpson et al., 2006). However, when they encounter a food dish containing protein-only artificial diet, they stop and feed for extended periods of time. This suggests dietary protein might be needed to sustain feeding.

Several GF studies have documented strong differences in macronutrient regulation in herbivores fed diets that were either extremely protein- or carbohydrate-biased and/or extremely low in total macronutrient content. For example, Lee et al. (2002) explored the effects of diet p:c ratio and total macronutrient concentration on protein and carbohydrate regulation in a caterpillar species (*Spodoptera littoralis*). They

showed that macronutrient regulation was significantly affected by food total macronutrient concentration and that larvae increased protein consumption when offered dilute diets (25.2% total macronutrients versus 42%). Likewise, Le Gall and Behmer (2014) performed a choice experiment using a grasshopper species (*Melanoplus differentialis*) and showed that individuals prioritized dietary protein when the total macronutrient content of the diets were low. Taken together, these results suggest that decision rules for nutrient regulation on extreme diets may not be indicative of regulation across less extreme diets. The fact that protein is often prioritized in sub-optimal nutritional situations may indicate its importance in signaling an acceptable food source. This is particularly true given that the balance of multiple nutrients, rather than simply the concentration of total nutrients, can greatly impact herbivore feeding via chemosensory stimuli (Simpson and Raubenheimer, 1993) and has also been shown to impact the regulation of digestive enzymes in the gut, particularly in *H. zea*'s sister species *H. armigera* (Kotkar et al., 2009; Clissold et al., 2010; Sarate et al., 2012).

Despite detecting regulation for a specific protein-carbohydrate IT, when we reared neonates on diets with a range of different p:c ratios and total macronutrient concentrations (Figure 4.1b) we observed few differences in performance, and those that did occur were relatively small. Waldbauer et al. (1984) also ran a no-choice experiment from neonate to pupation, but only tested a p1:c1 and p4:c1 diet. They reported higher survival and shorter developmental time for larvae on the protein-biased diet; however, they did not see a difference in pupal wet mass. Unlike the Waldbauer et al. (1984) study, we saw no differences in survival across all diets (we had very low mortality

overall), and on the diets that contained 42% macronutrient content (the Waldbauer et al. (1984) diets had 43% total macronutrient content), no difference in developmental time was observed. Our results are also very similar to what has been found in other GF studies. For example, in caterpillars (Lee et al., 2002, 2003; Lee, Raubenheimer, and Simpson, 2004; Lee, Simpson, and Raubenheimer, 2004; Depland and Noseworthy, 2006; Lee, Behmer, and Simpson, 2006; Lee, 2007, 2010; Lee, Kwon, and Roh, 2012; Roeder and Behmer, 2014) and nymphal grasshoppers (Raubenheimer and Simpson, 2003; Behmer and Joern 2008; Le Gall and Behmer 2014) developmental time increases on very carbohydrate-biased diets. In addition, GF studies often report the lowest pupal mass for caterpillars and adult mass for grasshoppers reared on protein-biased diets, largely due to the fact that insects on protein-biased diets are lean, and contain very low lipid levels, due to low dietary carbohydrate concentration (Lee et al., 2002; Lee et al., 2003; Lee, Raubenheimer, and Simpson, 2004; Lee, Simpson, and Raubenheimer, 2004; Lee, 2010; Lee, Kwon, and Roh, 2012; Roeder and Behmer, 2014).

Roeder and Behmer (2014) measured larval, pupal, and reproductive performance of *H. virescens* (a close relative of *H. zea*) on artificial diets with different p:c ratios, and they too found larval performance results similar to those reported in the current study. However, they did show that diet p:c ratio had a significant negative effect on eclosion success and time to eclosion, especially when the p:c ratio of the test diets diverged strongly away from the IT. Additionally, they showed that when larval, pupal and reproductive performance was integrated, and extrapolated to the population level, insects reared on diets that most closely matched the IT generated the largest

populations, and had the shortest generation times. Collectively, the Roeder and Behmer (2014) results suggest that larval performance is, at best, a weak indicator of fitness for lepidopterans. Exploring the effects of food protein-carbohydrate ratio and total macronutrient concentration on *H. zea* over its entire lifetime would more fully characterize latent nutritional effects.

Nutrition impacts a herbivore's ability to deal with a range of stressors, including plant secondary compounds (Simpson and Raubenheimer, 2001; Behmer et al., 2002), pathogens (Lee et al., 2006; Lee et al., 2008; Ponton et al., 2011), and pesticides (Gordon, 1961). Insects are also capable of modifying their feeding behavior and resulting macronutrient intake to mitigate the effects of these stresses (Simpson and Raubenheimer, 2009; Behmer 2009). Having accurate nutrient regulation data for important economic pests such as *H. zea* is valuable because it provides a reference point for understanding their feeding behavior in the field. This in turn enables predictions to be made related to movement and distributions, and can help us anticipate how this species will respond to different control methods including, but not limited to, pesticides, transgenic plants (e.g., *Bt*), and biological control agents such as the fungal entomopathogen *Beauveria bassiana*. Up-dating and providing a more accurate account of how *H. zea* responds to different nutritional environments also provides a stronger foundation for further exploring its physiology and nutritional ecology. For example, the use of more realistic artificial diets in laboratory studies can be used to standardize nutritional environments across different physiological experiments, or enhance the

ecological relevance of applied studies such as diet-based resistance monitoring programs (e.g., Ali et al., 2006; Luttrell and Jackson, 2012).

Acknowledgements

We would like to thank all those who have contributed to this project by providing methodological, analytical, or editorial feedback. In particular, we would like to thank Xiangfeng Jing, Marion Le Gall, Paul Lenhart, Cesar Valencia, and Rebecca Clark. We would also like to acknowledge our funding sources for this project, including the C. Everette Salyer Fellowship (Texas A&M Entomology Department), the Charles R. Parencia Endowment (Texas A&M Entomology Department), and this research was partially supported by an NRI-CSREES-USDA grant (#2007-35302-18185) to Spencer Behmer.

CHAPTER V

A SIGNIFICANT ROLE FOR DIETARY PROTEIN AND CARBOHYDRATES ON CRY1AC SUSCEPTIBILITY IN *HELICOVERPA ZEA* (LEPIDOPTERA: NOCTUIDAE): THE IMPORTANCE OF PLASTICITY IN RESISTANCE MONITORING

Overview

Incidents of insecticide resistance in agricultural pest species represent a costly and ever-increasing problem that poses significant challenges for meeting the nutritional demands of our growing global population. The development and spread of genetic mutations conferring resistance is the primary explanation for observations of low pesticide efficacy and population outbreaks in agricultural systems. As a result, the potential for gene-by-environment interactions, or plasticity, to play a dominant role in mediating pesticide tolerance is often overlooked. Nutrition is an environmental factor that has the potentially to strong impact susceptibility. Macronutrients such as protein (P) and carbohydrates (C) are highly variable across plant tissues and have been shown to strongly affect insect behavior, physiology, and performance, including detoxification potential. In this study, we used cotton as a model system to explore the potential for dietary macronutrients to impact susceptibility of the cotton bollworm *Helicoverpa zea* to Cry1Ac proteins found in *Bt* cotton. Using artificial diets, we mimicked the macronutrient content of different cotton tissues and reared larvae on diets with various concentrations of Cry1Ac. We found that diet macronutrients had significant effects on

susceptibility to Cry1Ac. Specifically, P:C ratio had strong effects on larval performance at sub-lethal doses, while total macronutrient concentration had the strongest impact on survival at higher doses. Overall, larvae performed best when reared on the diet that match the self-selected intake target for this species (P:C of 1.6:1). High-C diets produced the worst survival and performance. These results have important implications for resistance management, as many rearing diets for *H. zea* are C-biased, and using a sub-optimal diet such as this could lead to spurious results in the resistance bioassays used to detect resistance.

Introduction

Acreage of *Bt* crops has increased by over 60-fold since its introduction in 1996, with Bt acreage exceeding 1 billion acres throughout the world (Tabashnik et al., 2013). With this increased usage, reports of insect resistance to *Bt* is also increasing. Currently, incidents of field-evolved resistance to *Bt* has been reported in 5 of the 13 pest species examined (Tabashnik et al., 2013). The overriding assumption in *Bt* resistance monitoring is that genetic factors are primarily responsible for the presence of resistant phenotypes (Tabashnik et al., 1994; Moar et al., 2008). This is the case despite the fact that genetic mutations are rarely substantiated after reports of reduced susceptibility to *Bt* crops is observed in the field. It is ultimately the expression of specific genes that is responsible for producing a resistant phenotype, and importantly, the expression of all genes are, at least to some extent, dependent on environmental factors. Phenotypic plasticity, or the ability of a single genotype to produce a range of phenotypes across

different environmental conditions, is widespread across traits and organisms (Travis 1994; West-Eberhard 2003). Plasticity occurs due to interactions between genes and environmental factors, producing a range of phenotypic responses (Schlichting and Pigliucci, 1998; Pigliucci, 2001; Gibson, 2008). Figure 5.1 is a reaction-norm model that displays the genetic and environmental components of a phenotypic trait and how the interaction between these two components can produce plasticity (Figure 5.1b). Despite the pervasiveness of phenotypic plasticity, it is rarely incorporated into evolutionary models (Chevin et al., 2010), due to the complexity of incorporating interactions and limitations on available information regarding the regulation of complex traits.

From an evolutionary perspective, plasticity is likely to occur when populations experience a variable environment and when the fitness advantage of specific phenotypes vary across different environments (Bradshaw 1965; Levins 1968; Via & Lande 1985; Lively 1986; Gomulkiewicz &

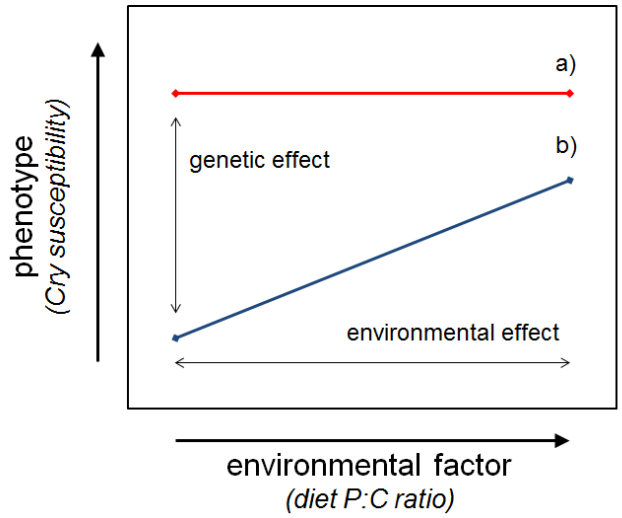

Figure 5. 1. The reaction norm for the phenotype of two genotypes across an environmental factor. Genotype (a) has the same phenotype across environments, indicating no plasticity, while genotype (b) shows different phenotypes depending on the the environment, indicating a plastic response. Phenotype differences between (a) and (b) (y-axis) in any given environment are due solely to differences in genotype, while differences in phenotype along the x-axis are due to environmental effects.

Kirkpatrick 1992; Moran 1992; Ghalambor et al. 2007). Plant nutrient content is an environmental factor that meets both these criteria. Across plant-insect interactions, plant nutrient content is often highly variable (Güsewell, 2004; Schoonhoven et al., 2005; Deans et al., 2015), meaning that most insect herbivores forage in a heterogeneous nutritional landscape that displays a gradation of nutritional optimality. This variation can have important implications for both feeding behavior and performance, because insects are able to detect differences in plant macronutrients (sugars and amino acids) and actively regulate their intake of protein (P) and carbohydrates (C) to meet their physiological requirements (Schoonhoven, 1968; Bernays and Chapman, 1994; Behmer, 2009). Also, the nutritional quality of plant resources, particularly the concentration and balance of P and C, has been shown to have strong effects on insect performance, including growth rate and reproduction in laboratory studies (Simpson and Raubenheimer, 1995; Behmer, 2009), as well as also detoxification ability (Simpson and Raubenheimer, 2001; Behmer, Simpson, Raubenheimer, 2002). Despite the evidence that plant macronutrients are highly variable and that they have strong impacts on detoxification in insect lab studies, the effect of nutrition on insecticide susceptibility/resistance is not well understood, particularly in agricultural systems where nutritionally-mediated resistance may have significant economic implications (Shikano and Cory, 2013; Shikano and Cory, 2014; Orpet et al., 2015).

In this study we used *Helicoverpa zea* as a model to explore how plant macronutrients impact susceptibility to Cry1Ac endotoxins, one of the major plant-incorporated insecticides widely expressed in *Bt* cotton. The objectives of this study

were to determine how macronutrient concentration, as well as protein-to-carbohydrate ratio (P:C), of different cotton tissues affected survival and performance across lethal and sub-lethal concentrations of Cry1Ac. We hypothesized that nutrition would have strong effects and that the diets that most closely match the optimal diet for *H. zea,* i.e., the self-selected intake target (IT), would confer the greatest survival and performance for insects challenged by Cry proteins in their diet. Waldbauer et al. (1984) reported the IT for this species to be highly protein-biased, with a P:C of 4:1, but the IT for *H. zea* was recently re-assessed by Deans et al. (*submitted*). Using a geometric framework they found the IT to be 1.6:1, which is more inline with the ITs found for other lepidopteran species, and very close to that of *H. zea*'s close relative *Heliothis virescens* (Behmer, 2009). A few studies have explored the effects of diet on susceptibility to Cry endotoxins in different lepidopteran species (Sayyed et al., 2003; Blanco et al., 2009; Shikano and Cory, 2013; Shikano and Cory, 2014; Orpet et al., 2015). However, the majority of these studies were focused on differences in nutritional effects on resistant versus susceptibility strains and did not considered nutritional regulation on the part of the insect in the context of ecologically-relevant diets. In addition, most of these studies used Cry sources that are less relevant to *Bt*-plants, such as spore/protoxin mixtures and encapsulated protoxin. This study will be the first, to our knowledge, to focus specifically on how nutrition affects susceptibility due to phenotypic plasticity.

Methods

Insects

H. zea eggs were purchased from Benzon Research (Carlisle, PA). Upon hatching, neonates were individually placed, using a fine-tipped paint brush, into 1 oz. clear condiment cups with mesh lids. Each cup contained one of twelve experimental diets that varied in protein-to-carbohydrate ratio (P:C) and Cry1Ac concentration (see below). All individuals were kept in a growth chamber (Model I-66VL; Percival Scientific, Perry, IA, USA) set at 25°C with a 14:10 L:D cycle for the duration of each experiment.

Artificial Diet

A synthetic diet, originally developed by Ritter and Nes (1981) and then later modified as described by Jing et al. (2013), was used. The key ingredients were vitamin-free casein, sucrose, cellulose, Wesson's salt mix, Torula yeast, lipids (cholesterol, linoleic and linolenic acid) and vitamins. In total, 4 different diets that had unique protein (P) and digestible carbohydrate (C) profiles were made. All other ingredients, except for cellulose, were held constant between the different diets; the amount of cellulose in a diet varied inversely with total macronutrient content. All of the experimental diets were mixed with a 1% agar solution to provide caterpillars with both nutrients and water.

To maintain relevance to natural conditions, the P and C content, as well as the total macronutrient concentrations (P+C), of our experimental diets were selected to

match values documented for different cotton tissues, as in Deans et al. (*in press*). Cotton is a common resource for *H. zea* larvae, as a result, larvae are likely to encounter resources of this quality in a natural setting. We tested three diet P:C ratios, which contained a total macronutrient concentration (P+C) of 42% (by dry mass). One diet was C-biased (12p:30c), one approximated the published intake target for *H. zea* (24p:18c) (Deans et al., *submitted*), and one was P-biased (30p:12c). We also tested a diet that matched the intake target, but contained 68% total macronutrients (39:29). Table 5.1 shows each experimental diet and the corresponding cotton tissue it references.

Cry Solutions

Trypsin-activated HPLC purified Cry1Ac was purchased from the Department of Biochemistry at Case Western Reserve University (Cleveland, OH) and stored at -80°C. Four different Cry1Ac stock solutions were prepared for incorporation into the experimental diets and were stored at -20°C until diet preparation. Because Cry endotoxins degrade at room temperature over time, each artificial diet was refrigerated and then mixed with the appropriate amount of Cry stock concentration just before feeding, and larvae were fed fresh diets a minimum of every four days.

The concentrations of Cry stock solutions were standardized so that the same amount of liquid was needed to achieve the desired Cry concentration in the diets. This controlled for the amount of water being added to the diets across Cry treatments. During diet preparation, the total amount of diet needed to feed all replicates within a single treatment was weighed and calculations were done to determine the exact amount

of the corresponding Cry1Ac stock solution needed to achieve the overall Cry1Ac concentration in the diet (ug of Cry per g of diet). Stock solutions were thawed, the appropriate amounts were added to each diet, and the diet was thoroughly mixed before being portioned into each rearing container. Water, rather than Cry solution, was added to the control diets in the same amounts as the Cry treatments to account the addition of water across Cry treatments. In total, there were four different Cry1Ac concentrations tested: two concentrations produced high mortality in preliminary studies on our lab-reared strain of *H. zea* (hereto referred to as "lethal" concentrations) and two concentrations that produced low mortality (hereto referred to as "sub-lethal" concentrations). The lethal concentrations were 1.0 ppm and 3.0 ppm and the sub-lethal were 0.1 ppm and 0.6 ppm.

Experimental Protocol

One experiment was conducted that tested all four Cry concentrations on each diet. Larvae were fed on their respective treatment diets from hatching and were monitored through eclosion as adults. Survival was documented for all treatments, but performance, including developmental time, pupal mass, eclosion success, and consumption were only measured for surviving larvae. Consumption of each diet was measured by obtaining the wet and dry mass of each diet portion and using a wet-dry mass regression calculated separately to determine differences in initial and final dry mass. The consumption and frass data were used to calculate assimilation efficiency (%

of food ingested that is not egested) and net growth efficiency (% of assimilated food that goes toward growth) across treatments.

Data Analysis

A Kaplan-Meier survival analysis (Mantel-Cox test) was used to determine differences in larval survival distributions and developmental time (time to pupation) across treatments. This allowed us to look for the main effects of diet, Cry1Ac concentrations, and interactions between the two. A two-way ANOVA was used to determine differences in pupal mass, consumption, assimilation efficiency, and net growth efficiency across treatments. A logistic regression was used to determine the effects of diet ad Cry concentration on eclosion success. All analyses were done using SPSS version 21 for Windows (SPSS Inc., Chicago, IL, USA).

Results

Survival

There was a strong interactive effect of diet and Cry1Ac concentration on caterpillar survival (Table 5.1). Across the control treatments, which contained no Cry, 96% of larvae survived to pupation. Survival dropped significantly in the other treatments as the concentration of Cry increased. The 0.1, 0.6, 1, and 3 ppm treatments showed 88.2%, 35.5%, 8.7%, and 0% survival respectively (Fig. 5.2 and Fig. 5.3). The Kaplan-Meier results showed significant effects of diet on survival in the treatments with the two highest Cry concentrations of 1 ppm (Mantel-Cox, $p<0.0001$) and 3 ppm

(Mantel-Cox, p<0.0001). Across the controls, there was no significant effect of diet on survival (Mantel-Cox, p=0.092) (Fig 5.2a); however, when 1 ppm of Cry1Ac was incorporated into the diets, the 24:18 and the 39:29 diets showed significantly higher survival than both the C-biased 12:30 diet and the P-biased diet of 30:12, which had similar survival curves (Figure 5.2b). These results indicate a strong effect of P:C ratio on survival, as both the 24:18 and 39:29 diets have the same ratio but different total macronutrient concentrations, and show that survival is the highest on the diets that most closely match the IT for this species. At 3 ppm, the 39:29 diet again showed the highest survival, while larvae in the other three diet treatments had significantly reduced survival (Fig 5.2c). These results suggest that total macronutrient concentration is important for survival at 3 ppm, as the

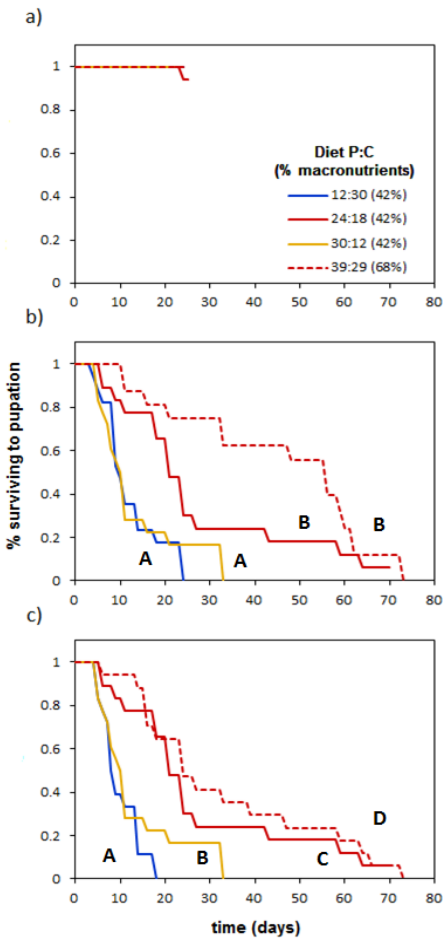

Figure 5. 2. Survival plots for larvae on each diet P:C when (a) no Cry is present (p=0.092), at (b) 1 ppm (p=<0.0001), and at (c) 3 ppm (p<0.0001). Line endpoints indicate that either all individuals died or pupated. Different letters indicate post hoc differences at the p=0.05 α level.

68% total macronutrient diet of 39:29 produced much higher survival than the other three, which had only 42% total macronutrient content.

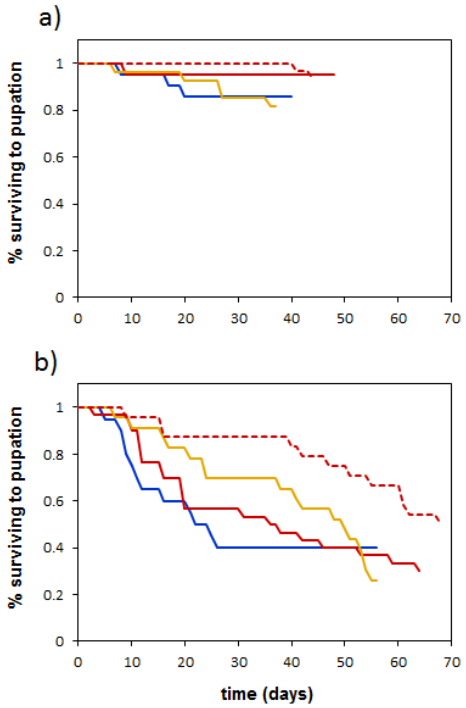

Figure 5. 3. Survival curves for each diet at sub-lethal 0.1 ppm (a) and 0.6 ppm (b) Cry concentrations. There was no significant effect of diet on survival at either concentration.

Developmental Time

Table 5.1 shows that Cry1Ac concentration had a strong impact on developmental time across treatments (Mantel-Cox, p<00001). Larvae from the control treatments took an average of 20.4 days to pupate, while those surviving in the 0.1, 0.6, and 1 ppm treatments took an average of 31.8, 45.0, and 44.0 days respectively. No larvae survived to pupation in the 3 ppm treatments. Diet did not have a significant effect on time to pupation for larvae in the controls (Mantel-Cox, p=0.779) (Fig 5.3a), 0.1 ppm (Mantel-Cox, p=0.588) (Fig 5.3b), or 1 ppm (Mantel-Cox, p=0.470) treatments; however, there was a significant diet effect for the 0.6 ppm treatment (Mantel-Cox, p=0.004) (Fig 5.3c). At 0.6 ppm, larva on the 24:18 and 39:29 diets had the fastest developmental times, followed by the P-biased 30:12 diet, and the C-biased 12:30 diet. These data indicate a strong effect of diet P:C ratio, as larva reared on the diets that most closely match the IT

for this species had significantly faster developmental times.

Pupal Mass

Table 5.2 shows that there was a significant effect of Cry concentration on pupal mass ($F_{3, 241}$=38.97, p<0.0001), but no diet effect ($F_{3, 241}$= 0.05, p=0.985). High mortality prevented including the 1 ppm and 3 ppm treatments in the analysis, but comparisons between the controls, 0.1 ppm, and 0.6 ppm treatments showed that pupal mass decreased significantly as Cry concentration increased (Fig 5.3d-f). Controls showed an average pupal wet mass of 315.7 mg, while the average for the 0.1 ppm treatment was 264.1 mg and that for the 0.6 ppm treatment was 216.9 mg.

Eclosion Success

There was a significant effect of diet (X^2= 34.44, df=3, p<0.0001) and Cry concentration (X^2= 6.39, df=2, p=0.041) on eclosion success, but no significant diet*[cry] interaction. Table 5.3 shows that the percentage of adults exhibiting wing deformations upon eclosion was significantly higher on the 39:29 diet in comparison to the other 42% macronutrient diets which had a low percentage of deformities (Fig. 5.4g-i). Deformities were also higher in the 0.6 ppm treatment compared to the controls. This indicates that diet and Cry concentration can significantly impact adult performance. Although the full extent to which wing deformities affect overall fitness is unknown, impaired flight should have major negative consequences.

Table 5.1. Results of the Kaplan-Meier analysis (Mantel-Cox) testing the effect of diet on survival and developmental time across Cry concentrations.

	Chi-Square	df	Significance
Survival			
0 ppm (controls)	6.44	3	0.092
0.1 ppm	3.10	3	0.377
0.6 ppm	4.23	3	0.238
1.0 ppm	24.34	3	<0.0001
3.0 ppm	51.48	3	<0.0001
Developmental Time			
0 ppm (controls)	1.09	3	0.779
0.1 ppm	1.93	3	0.588
0.6 ppm	13.49	3	0.004

Table 5.2. Two-way ANOVA results for the effects of diet and Cry concentration on pupal mass.

Variable	Source	F Statistic	df	Significance
pupal mass	model	13.33	11	<0.0001
	diet	0.50	3	0.985
	[cry]	38.97	2	<0.0001
	diet*[cry]	0.84	6	0.539

Table 5.3. Logistic regression results for the effects of diet and Cry concentration on the percentage of normally eclosed adults (no wing deformation).

Variable	Comparisons	X^2	df	Significance
diet		34.44	3	<0.0001
	39:29 vs 12:30	23.02	1	<0.0001
	39:29 vs 24:18	25.31	1	<0.0001
	39:29 vs 30:12	22.36	1	<0.0001
[cry]		6.39	2	0.041
	control vs 0.1 ppm	2.54	1	0.111
	control vs. 0.6 ppm	6.34	1	0.012
	0.1 ppm vs 0.6 ppm	2.26	1	0.133
diet*[cry]		8.13	6	0.23

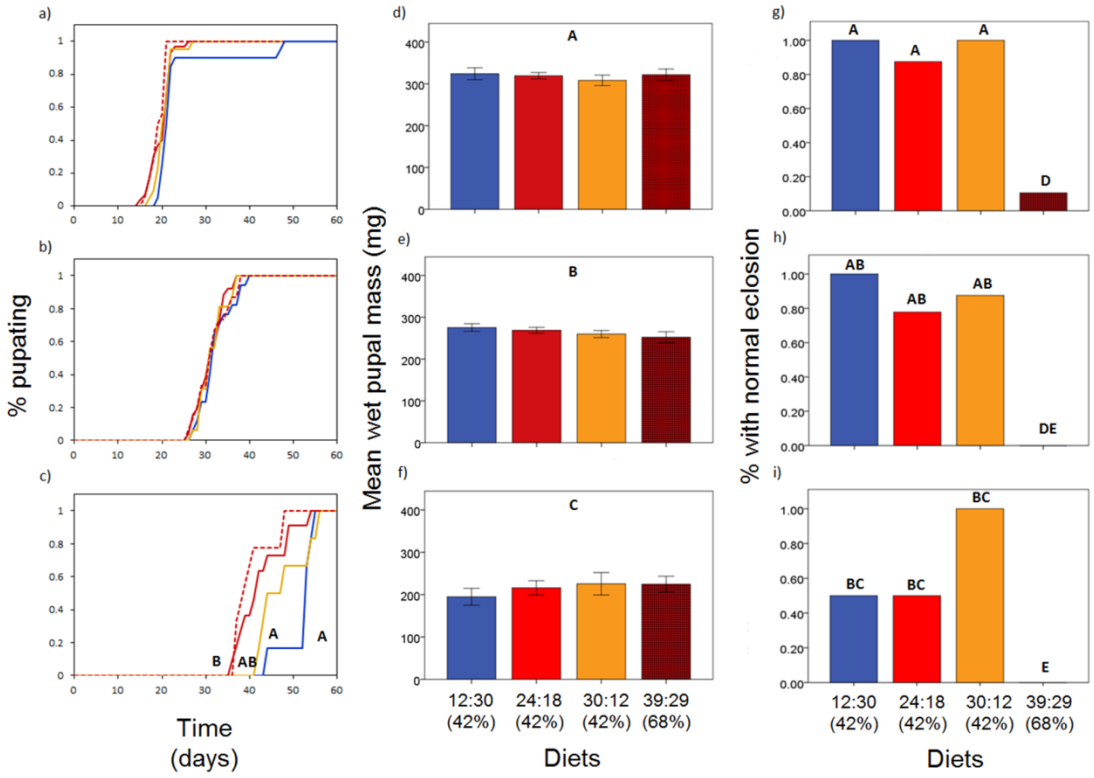

Figure 5. 4. Performance parameters for all diets across controls (no Cry) (first row; a, d, g), 0.1 ppm (second row; b, e, h), and 0.6 ppm (third row; c, f, i). Panels a-c show developmental time, d-f shows pupal mass, and g-i eclosion success. A significant diet effect was found for development time at the 0.6 pp concentration only ($p=0.004$). A significant Cry effect was found for pupal mass ($p<0.0001$), and a significant diet ($p<0.0001$) and Cry (0.041) effects were found for eclosion success. Different letters indicate post hoc differences at the $p=0.05$ α level.

Consumption

There were significant interactions between diet P:C and Cry concentration (sub-lethal For the 12:30 and 39:29 diets, consumption was the highest on the 0.1 ppm treatments concentrations only, as consumption was not measured for the 1 ppm and 3 ppm treatments due to high mortality) on both total and mass-specific consumption (Table 5.4). Figure 5.5a shows that total consumption was significantly higher in the Cry

treatments versus the controls. For diet 24:18 ($p<0.0001$) and 30:12 ($p=0.002$) consumption was only significantly higher for the 0.1 ppm treatments but not the 0.6 ppm treatments. but intermediate for the 0.6 ppm treatments (Table 5.6). Across diets there was no effect of total macronutrient concentration on consumption, as consumption on the 39:29 was similar to all the other diets, but across the 42% macronutrient diets consumption on the C-biased 12:30 diet was significantly higher than the 24:18 diet ($p=0.008$) at 0.1 ppm. At 0.6 ppm, consumption on the 42% macronutrient diets was similar, while the 39:29 diet showed the lowest consumption, which was only significantly lower than the 30:12 diet ($p=0.032$).

Table 5.4 also shows that when accounting for differences in developmental time and insect mass, we also found a significant interaction between diet and Cry concentration on mass-specific consumption. Despite differences in total consumption, Figure 5.6a shows that Cry concentration did not have significant impacts on mass-specific consumption on any diets except 39:29, which did show significantly higher mass-specific consumption on the 0.1 ppm treatment. Also at 0.1 ppm, the 39:29 diet had significantly higher mass-specific consumption than the 24:18 diet ($p=0.021$), which showed the lowest mass-specific consumption. Again, these trends corresponded strongly to the mass-specific amounts of Cry1Ac consumed throughout the experiment (Fig. 5.6b). Comparisons between total and mass-specific consumption suggest that most of the differences in total consumption were due to differences in developmental time and did not results from an increase in feeding rate.

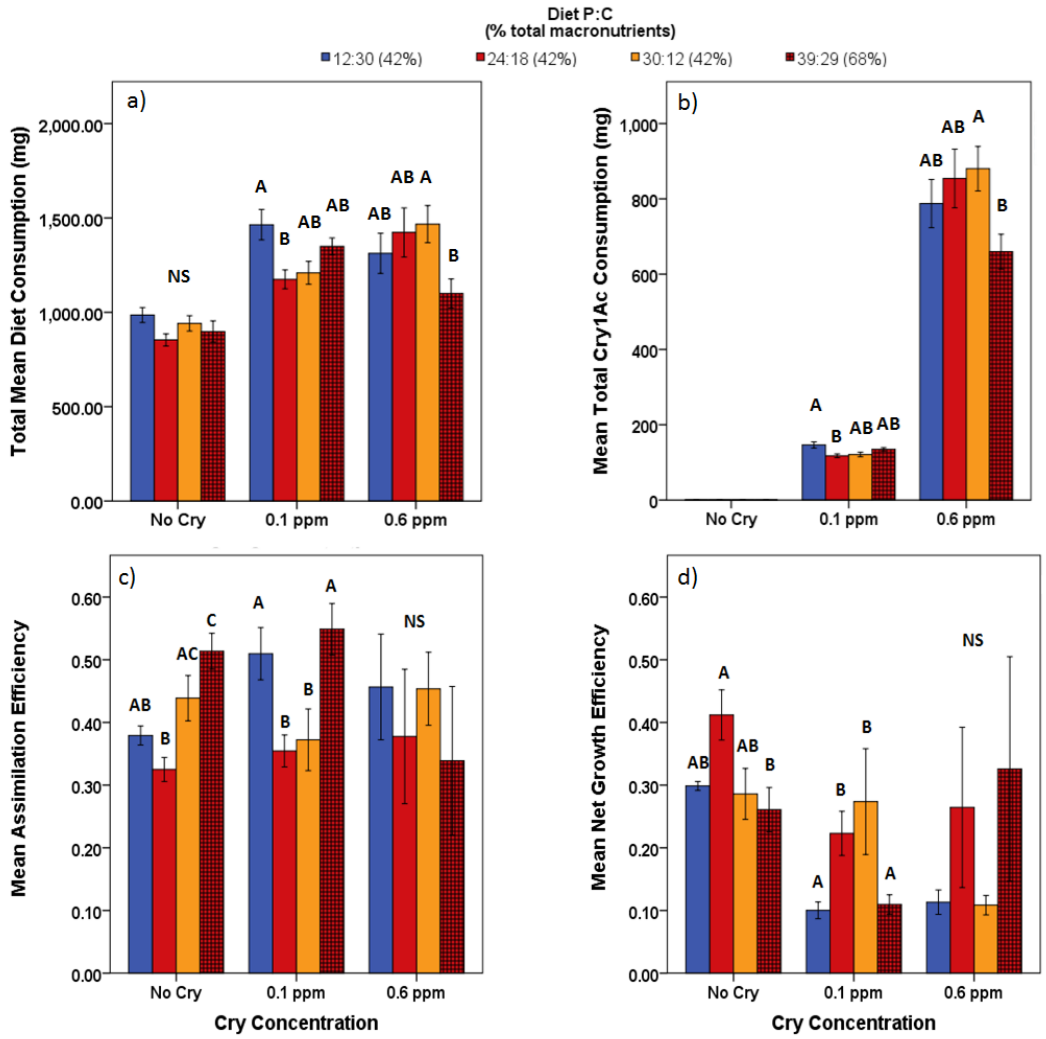

Figure 5. 5. Total diet consumption (a) and total Cry1Ac consumption (b) across diets for the controls (no Cry), 0.1 ppm, and 0.6 ppm concentrations. The second row show mean assimilation efficiency (c) (AE = mass of food consumed- mass of feces) and mean net growth efficiency (d) (NGE = mass gained/AE). There were significant interactions between diet*[cry] for total consumption (p=0.014), total Cry1Ac consumption (p=0.002), assimilation efficiency (p=0.043), and net growth efficiency (p=0.002). Different letters indicate post hoc differences at the p=0.05 α level.

Table 5.4. Two-way ANOVA results for differences in total and mass-specific diet and Cry1Ac consumption, as well as assimilation efficiency and net growth efficiency. Consumption was only measured for the controls and the 0.1 ppm and 0.6 ppm concentration treatments. Controls contained no Cry1Ac, so Cry1Ac consumption analyses only compare the 0.1 ppm and 0.6 ppm treatments.

Variable	Source	F Statistic	df	Significance
total consumption	model	13.33	11	<0.0001
(mg)	diet	2.43	3	0.067
	[cry]	59.49	2	<0.0001
	diet*[cry]	2.76	6	0.014
mass-specific consumption	model	1.82	11	0.055
(mg/mg/day)	diet	0.27	3	0.850
	[cry]	3.99	2	0.020
	diet*[cry]	2.18	6	0.048
Cry1Ac consumption	model	224.15	7	<0.0001
(ng)	diet	1.25	3	0.295
	[cry]	1481.76	1	<0.0001
	diet*[cry]	5.22	3	0.002
mass-specific Cry1Ac consumption	model	80.27	7	<0.0001
(ng/mg/day)	diet	0.22	3	0.881
	[cry]	483.20	1	<0.0001
	diet*[cry]	2.94	3	0.038
assimilation efficiency	model	4.50	11	<0.0001
(% of food mass assimilated into	diet	3.49	3	0.018
insect biomass)	[cry]	1.08	2	0.345
	diet*[cry]	2.25	6	0.043
net growth efficiency	model	13.81	11	<0.0001
(% of assimilated food that goes	diet	4.15	3	0.008
towards growth)	[cry]	46.69	2	<0.0001
	diet*[cry]	3.66	6	0.002

Figure 5.5b shows that differences in total consumption had corresponding effects on the total amount of Cry1Ac ingested in each treatment. At 0.1 ppm, larvae on the 24:18

diet consumed the least amount of Cry1Ac in total, while those on the 12:30 diet consumed the most. Cry1Ac consumption on the 30:12 and 39:29 was intermediate. At 0.6 ppm, total Cry1Ac consumption was similar across all 42% macronutrient diets but substantially lower for the 39:29 diet, which was only statistically different from the 30:12 diet ($p=0.037$).

Mass-specific consumption of Cry1Ac also strongly corresponded to total mass-specific diet consumption. Figure 5.6b shows that larvae on the 42% macronutrient diets had similar feeding rates, while significant differences were apparent between the 24:18, which had the lowest mass-specific Cry1Ac consumption, and 39:29, which had the highest mass-specific Cry1Ac consumption. There were no differences across diets, however, at 0.6 ppm.

Digestibility Indices

Assimilation efficiency (AE), sometimes referred to as approximate digestibility, reports the percentage of diet ingested that is digested and retained by the caterpillar (i.e. not egested), while net growth efficiency (NGE), sometimes referred to as ECD (efficiency of conversion of digested (assimilated) food to biomass), indicates the percentage of digested food that is used for growth. Table 5.4 shows that there were significant interactions between diet P:C and Cry concentration on AE and NGE across the sub-lethal Cry concentrations. Post hoc results show that when no Cry was present AE was highest on the more concentrated 39:29 diet and lowest on the 24:18 diet (Fig. 5.5c). When

0.1 ppm of Cry was present, AE remained high on the 39:29 diet and increased for the C-biased 12:30 diet, while the 24:18 and 30:12 diets were about 10% lower. At 0.6 ppm

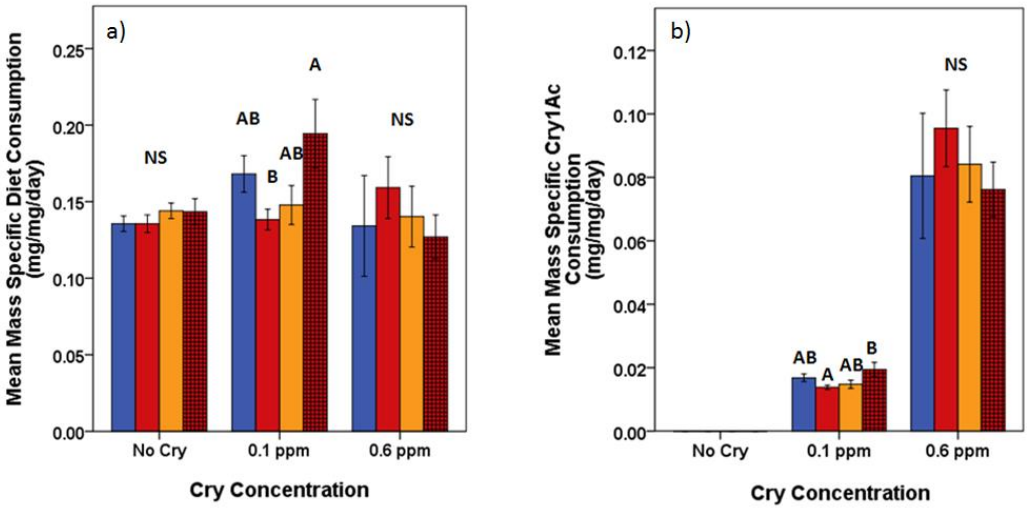

Figure 5. 6. Mass-specific diet consumption (a) and Cry1Ac consumption (b), which factors in differences in developmental time and mass gain across diets. There was a significant diet*[cry] interaction for both mass-specific consumption (p=0.048) and mass-specific Cry1Ac consumption (p=0.038). Different letters indicate post hoc differences at the p=0.05 α level.

Cry AE was rather variable and no differences between diets were detected (Fig. 5.5c). O verall, NGE showed an inverse relationship with AE. For the control treatments, NGE was highest for the 24:18 diet and lowest for the 39:29 diet. The 12:30 and 30:12 diets were intermediate (Fig. 5.5d). At 0.1 ppm the 24:18 and 30:12 diets had the highest NGE, while the 12:30 and 39:29 diets were about 10-15% lower. The NGEs at 0.6 ppm were again highly variable with no detectable differences between diets (Fig. 5.5d).

Discussion

H. zea exhibits significant nutrient-mediated plasticity in susceptibility to Cry1Ac. We found that both diet P:C and total macronutrient concentration had unique effects on susceptibility, but also that these effects were dependent on the concentration of Cry present. When no Cry1Ac was present, diet macronutrient composition had minimal impacts on survival and larval performance. This result is consistent with other nutritional studies on generalist caterpillars (Lee et al., 2002; Lee et al., 2006; Roeder and Behmer, 2014), including *H. zea* which is robust to variations in diet P:C during their larval stage (Deans et al., *submitted*). Roeder and Behmer (2014), however, do discuss the limitations of using larval performance to infer lifetime fitness. They found few effects of diet P:C on larval performance in *H. virescens*, but strong latent effects in the pupal and adult stages, including impacts on eclosion success, lifetime survival, and egg production. They concluded that that larval performance is an inaccurate indicator of overall fitness for lepidopterans. Our eclosion success data support this conclusion, as we saw little effect of diet on larval performance, but observed a drastically higher percentage of wing deformities on the macronutrient-rich 39:29 diet in the absence of Cry1Ac. Although we do not know the extent of fitness repercussions due to having wing deformities, it is reasonable to assume that individuals with this deformity would have less mating success than those without it.

When Cry was present, we found that larvae did significantly better when reared on the diets that most closely matched their IT. Although Waldbauer et al. (1984) originally inferred the IT for *H. zea* to be 4:1, a revised IT of 1.6:1 which is more inline

with other lepidopteran ITs was recently determined using a geometric framework (Deans et al., *submitted*). The 24:18 and 39:29 diets, which have a P:C ratio of 1.3:1, were the closest to the optimal diet for this species and showed the best survival and performance. Simpson and Raubeheimer (2001) reported similar results in locusts, where nymphs forced to consume diets with the grass allelochemical tannic acid had better survival and performance on the diet that represented the IT. We also found an interesting interaction between diet P:C and total macronutrient concentration. Although both the 24:18 and 39:29 diets, which have the same P:C ratio, showed the highest survival at 1 ppm of Cry1Ac, at higher Cry concentrations the more concentrated 39:29 diet produced the best survival and faster developmental times than the other 42% macronutrient diets. Taken together, these results suggest that diet P:C is important for survival at low Cry concentrations, but that total macronutrient content is most important at higher concentrations. We only tested one P:C ratio at 68% total macronutrients, so we cannot speak to the effect of diet P:C at higher total macronutrient concentrations; however, there is evidence that in lepidopterans total macronutrient content has stronger impacts at low P:C ratios under control conditions (Lee et al., 2002; Lee et al., 2004).

Despite observing the best larval survival and performance on the diets closest to the IT, when exploring performance beyond the larval period our data suggest that the diet with the best performance in the absence of Cry may not be optimal in presence of Cry. When Cry was present, larval survival and developmental time was consistently better for larvae on the high total macronutrient 39:29 diet at all Cry concentrations. Additionally, the 39:29 diet produced significantly higher survival at the highest

concentration of 3 ppm, indicating that this diet was overall the most optimal in the presence of Cry1Ac. In the absence of Cry, however, this diet showed a high proportion of wing deformation in adults across all treatments. As a result, this would make the 39:29 diet the least optimal diet under control conditions. These results show an interesting trade-off between diet optimality under stress and no-stress conditions. For *H. zea*, feeding on a diet that matches the IT is optimal under control conditions, but if the diet has a high total macronutrient concentration it could negatively impact adult fitness. A diet that matches the IT and has a high concentration of total macronutrients would, however, be considered optimal for this species in the presence of Cry, as more larvae would survive through eclosion despite a higher frequency of wing deformities.

When insects herbivores are not allowed to self-select their diets and are instead forced to feed on a diet that does not match their IT, feeding patterns often change in predictable ways. Compensatory feeding, where one macronutrient is over-ingested to meet requirements for another macronutrient, is a common mechanism for dealing with dietary imbalances (Raubenheimer and Simpson, 1993; Simpson and Raubenheimer, 1993; Lee et al., 2002; Behmer, 2009). Compensatory feeding is also common on diets with low total macronutrient concentrations, as it is necessary to eat more of a dilute food to meet the both energetic and macronutrient demands. Overall, generalist caterpillars show a weak propensity for compensatory feeding across diets that vary in P:C ratio (Deans et al., *submitted*; Lee et al., 2006), although in some cases caterpillars have been shown to prioritize P intake and over-consume a C-biased diet in order to reach their requirements for P (Lee et al., 2002). Generalist caterpillars do, however,

show strong compensation on dilute diets (Lee et al., 2004), exhibiting reduced consumption of foods with high total macronutrient concentrations and increased consumption of diets with low total macronutrient concentrations. Additionally, many studies have confirmed that several lepidopteran species, including *H. zea* (Gore et al., 2005), *H. armigera* (Zhang et al., 2004), *Heliothis virescens* (Gould et al., 1991; Benedict et al., 1992, 1993,; Parker and Luttrell, 1999), *Spodoptera exigua* (Berdegué et al., 1996), and *Trichoplusia ni* (Li et al., 2006), can detect the presence of Cry endotoxins in artificial diet and plants, and actively avoid ingesting them even when found at concentrations that cause low mortality. When feeding on a toxic resource, larvae must reconcile meeting their nutritional demands with minimizing the intake of the toxin. When forced to feed on a toxic resource (that is detectable), differences in larval consumption across diets that vary in nutritional optimality can indicate where these trade-offs occur.

In our study, there were few differences in consumption across diets under control conditions; however, at sub-lethal concentrations of Cry larvae reared on the 39:29 diet were capable of ingesting more Cry-containing diet, hence more overall Cry1Ac, while maintaining high survival and exhibiting superior performance. Mass-specific consumption of the 39:29 diet was also higher in the 0.1 ppm treatment than the control, indicating that larvae on this diet increased their consumption rate when Cry1Ac was present at this low concentration, potentially to obtain fuel for detoxification. At the higher 0.6 ppm concentration, consumption rate on the 39:29 diet dropped back down to control levels. At 0.1 ppm, mass-specific consumption on the 39:29 diet was also

significantly higher than the 24:18 diet, which has the same P:C ratio. This suggests that larvae were willing to consume more Cry-containing diet if it had a high concentration of total macronutrients, again pointing to an important role for macronutrient concentration and increased caloric content, i.e. energy, in potentially mitigating toxin exposure.

Although we observed no differences in consumption across control diets, we did see significant differences in post-ingestive utilization, which clearly indicate that the 24:18 diet (closest to the IT for *H. zea*) is the most optimal for growth under control conditions. Mean AE for the 24:18 diet was significantly lower than the 30:12 and the 39:29 diets; however, the NGE for the 24:18 was the highest. This shows that although a lower percentage of diet was assimilated in larvae fed the 24:18 diet, the percentage of assimilated food that was allocated to growth was the highest in comparison to the other diets. When 0.1 ppm of Cry was present we saw a significant increase in AE for the 12:30 diet, but no change for the other diets. The 12:30 and 39:29 diets had the highest AE, approaching 60%, while the 24:18 and 30:12 diets had the lowest efficiency, at around 35-37%. The increase in AE for the 12:30 in the presence of Cry is intriguing and may be a result of increased gut retention time to maximize P uptake from such a C-biased diet (Scriber and Slansky, 1981; Timmins et al., 1988; Slansky and Wheeler, 1989; Wheeler and Slansky, 1991; Lee et al., 2004). At 0.6 ppm there were no differences in AE or NGE between diets, but there was a significant drop in AE and a significant increase in NGE on the 39:29 diet between the 0.1 ppm and 0.6 ppm treatments, perhaps indicating a shift in optimality from the 24:18 diet to the 39:29 diet

at higher Cry concentrations (mortality was too high measure consumption at the 1 and 3 ppm concentrations).

Orpet et al. (2015) looked at the impact of diet P:C on susceptibility to Cry1Ac in two strains of *H. zea*, one selected for susceptibility and one that was selected to be genetically-resistant to Cry1Ac. Despite finding strong differences in survival and developmental time between strains, they didn't find any significant differences in survival across diet P:C when a LC_{50} dose of Cry1Ac was incorporated. There were also few differences in developmental time across diets for either strain in the presence of Cry1Ac. They did, however, find that when comparing mortality between the controls and the Cry1Ac treatments, the reduction in survival was more precipitous for the most P-biased diet (P:C ratio of 9:1) than the most C-biased diet (P:C ratio of 0.53:1) in the susceptible strain. The resistant strain did not show an interaction between diet P:C and survival in the presence of Cry1Ac.

The results of our study showed much stronger impacts of diet P:C on survival and performance. Differences between the results of Orpet et al. (2015) and our study could be due to several factors. First, the range of diet P:C ratios we tested, which was based on macronutrient concentrations and P:C ratios reported for different cotton tissues (Deans et al., *in prep*), was centered around the IT of 1.6:1 and was much narrower than those tested by Orpet et al. (2015). For instance our most P-biased diet had a P:C ratio of 2.5:1 and Orpet et al. (2015) tested two diets that were more extreme than this, a 4:1 and a 9:1. This was likely due to the fact that they cited the IT for *H. zea* as being 4:1, taken from Waldbauer et al. (1984), and likely wanted to test diet P:Cs

around this more P-biased ratio. Deans et al. (*submitted*) re-assessed the IT target for *H. zea* and found it to be much less P-biased than Waldbauer et al.'s (1984) original findings. Testing such a broad range of diet P:C ratios may have limited the ability of Orpet et al. (2015) to detect changes at a finer scale closer to the 1.6:1 IT. Also, given that we observed the most significant effects of diet on survival and performance across diet total macronutrient concentrations, Orpet et al. (2015) only tested diets at one macronutrient concentration, and was therefore unable to test this attribute of nutrition on Cry susceptibility.

Secondly, the source of Cry used could have had an impact on the results. Orpet et al. (2015) used a mixture of protoxin crystals and spores for part of the selection experiments, while MVPII, a hybrid protoxin encapsulated in *Pseudomonas fluorescens*, was used for the rest of the selection experiments and for the diet assays. In contrast, we used an activated form of Cry1Ac. Studies have shown that the relative activity of the protoxin form of Cry versus the activated form, which is more similar to the toxin expressed in transgenic *Bt*-plants, is highly variable (Tabashnik et al., 2011; Adang et al., 2014) and can also have varying effects on selection for resistance (Xu et al., 2005; Anilkumar et al., 2008). For instance, in Xu et al. (2005) selection for resistance to Cry1Ac in *H. armigera* using activated toxin produced a 564-fold increase in resistance, but this only resulted in a 5-fold increase in resistance to *B. thuringiensis* var. kurstaki itself.

Using spores or toxin encapsulated in bacterial cells would also likely trigger an immune response in the larvae, further complicating attempts to understand the

interaction between toxicity and diet quality. Although Cry endotoxins are derived from the bacteria *Bacillus thuringiensis*, the *cry* genes expressed in transgenic *Bt*-plants only produce the activated toxin, hence it is unlikely that feeding on Cry toxins expressed in transgenic plants would trigger immunity pathways, such as the production of anti-microbial peptides. Cunha et al. (2013) did find higher levels of phenoloxidase in *Spodotpera frugiperda* larvae fed on *Bt* cotton versus non-*Bt* cotton; however, hemocytes counts were lower in the *Bt* treatment and there were no differences in other indicators of cellular defense, such as total nitric oxide concentrations and total proteins in the hemolymph. Humoral and cellular immune defenses are resource-intensive, and several studies have shown that caterpillars alter their feeding behavior when infected with a pathogen, generally regulating for more dietary P (Lee et al., 2006; Lee et al., 2008; Povey et al., 2008; Cotter et al., 2010). It has also been shown that high-P diets improve survival and performance when infected (Lee et al., 2006; Lee et al., 2008; Povey et al., 2008; Cotter et al., 2010). Given these interactions, resource allocation to immune function in addition to detoxification could obscure the effects of diet on Cry susceptibility. While results based on the use of MVPII would be relevant to *Bt* sprays, which contain spores and protoxin, they may not be relevant to transgenic *Bt*-plants.

Lastly, it's possible that genetic differences between in the susceptible strain of *H. zea* used in Orpet et al. (2015) and the susceptible strain we tested may have impacted the results. The strain we used is highly inbred and would have possessed much lower genetic diversity compared to the field-derived strain tested in Orpet et al. (2015). It's possible that the higher genetic diversity in their strain may have produced a variable

response to the tested diet-Cry treatments, making trends harder to detect due to more genetic variation. It is also possible that different genotypes respond differently to nutritional variation in the presence of Cry. For example, although the differences were relatively small, Orpet et al. (2015) did find differences in how diet P:C impacted developmental time in the presence of Cry across the resistant and susceptible strains. They also reported significant effects of strain on sex-specific pupal mass across diets. Shikano and Cory (2014) found a similar effect of diet on *Bt*-related mortality for a susceptible and resistant strain of *Trichoplusia ni*, where the survival of the susceptible strain increased as P:C ratio increased, while the resistant strain had higher mortality on the most P-biased diet. It should be noted, however, that Shikano and Cory (2014) also used a spore and protoxin formulation rather than activated toxin, making comparisons between our study and theirs more tenuous (see above). These results indicate that genetic background may alter the impact that diet has on susceptibility to Cry; however, more strains will need to be tested in a standardized manner to fully understand the role of genetic background on nutritional plasticity in the presence of Cry endotoxins.

Our results have shown that diet macronutrient content has the ability to significantly impact susceptibility to Cry1Ac in *H. zea*. These data show that susceptibility to Cry endotoxins is plastic, and not genetically-determined in the strain we tested, supporting the concept that the underlying factors involved in susceptibility can be impacted by interactions between genetic and environmental factors, such as nutrition. The role of insect resistance monitoring (IRM) programs is to provide early detection of resistant alleles among pest populations that reduce susceptibility to the Cry

endotoxins produced by *Bt*-plants in order to minimize agricultural loses resulting from the reduced efficacy of this widely-used technology. IRM has an exclusive focus on the genetic components of resistance, particularly the detection of resistance alleles, their spread and persistence throughout pest populations, and the evolution of these genes. Although environmentally-mediated effects on susceptibility, i.e. plasticity and gene regulation, are not currently taken into account in IRM, they can have significant impacts on both broadscale pesticide failures, which have significant impacts on growers apart from genetic resistance, and they can influence the evolution of resistance itself. Therefore, there are two primary ways in which the results of this study relate to IRM.

First, nutrition does impact susceptibility, hence replicating the nutritional conditions that larvae encounter in the field when performing resistance bioassays is important for obtaining accurate results. Across the four studies that have reported field resistance in cotton for *H. zea*, two used a pinto bean-based diet (Ali et al., 2006; Ali et al., 2007), one used a wheat germ-based diet (Luttrell, 1999), and the other failed to mention the diet used (Luttrell and Ali, 2007). Other monitoring agencies use still other commercially-available diets from Frontier Agricultural Sciences (formerly Bio-Serv, Newark, DE) and Soutland Products Inc. (Lake Village, AR). Despite having different plant components, these diets do have rather similar macronutrient profiles; the pinto bean diet (Burton, 1969), the wheat germ diet (King et al., 1985), and the Bio-Serv diet have P:C ratios and total macronutrient concentrations of 0.5 (76.5%), 0.46 (64.4%), and 0.4 (63.2%) respectively. However, all of these diets contain P:C ratios that are very C-biased and very different from the self-selected optimal diet for *H. zea*, which has a

P:C of 1.6:1. These diets are relatively inexpensive and likely appear to be suitable rearing diets, particularly since the survival and performance of *H. zea* and closely related species are robust to nutritional variability under control conditions (Fig. 5.2a, Fig. 5.4a,d) (Deans et al., *submitted*; Roeder and Behmer, 2014). However, data from this study show that when Cry1Ac is present, C-biased diets such as these produce the lowest larval survival and performance, particularly in comparison to diets that more closely match the IT. This suggests that C-biased diets are nutritionally sub-optimal. Our data suggest that the discrepancy in macronutrients between the diet larvae are feeding on in the field and the diet used in resistance bioassays in the lab will likely confound the results of these resistance monitoring studies. In this case, larvae persisting on *Bt* crops could appear resistant in the field, but the same larvae would look susceptible in lab bioassays, simply due to testing them on a sub-optimal diet. Given our data, results from resistance bioassays that use these C-biased diets may overestimate susceptibility, ultimately underestimating resistance in the field and reducing our ability to detect resistant alleles if present.

Secondly, plasticity can impact susceptibility even in genetically-resistant strains and can also influence the evolution of resistant traits. Possessing a resistant allele isn't enough to ensure a resistant phenotype, because it is ultimately the expression of a gene, and often several genes, that produce a phenotype. It is at this junction where environmental factors can have an impact, causing variation in phenotypes. In this way, plasticity can increase the diversity of phenotypes upon which natural selection can act. The presence of plasticity for one phenotype may also have associated changes in others

due to physiological trade-offs, affecting morphological, behavioral, of developmental traits. These changes may then impact assortative mating and reproductive isolation, leading to speciation. For example, nutritional plasticity may allow a species to expand its host range, which could then have repercussions on mating and reproductive isolation, impacting speciation, as in the case of *Rhagoletis 111lternate* (Leclaire and Brandle, 1994). In some cases plasticity has been hypothesized to speed up speciation by providing a diversity of traits, while in other cases it has been hypothesized to slow down evolution due to the ambiguity between the phenotype and underlying genotype (Pigliucci, 2001; Price et al., 2003). The impact of plasticity on macroevolution is highly dependent on the life history of the organism in question, with few universal trends currently supported (Pigliucci, 2001). In terms of resistance, acknowledging the role of plasticity will be important, not only for detecting resistance in the field, but also for understanding how resistance evolves and can be maintained.

Acknowledgements

I would like to thank all those who have assisted in completing this project, either through providing technical assistance or general feedback, including but not limited to: all the members of the Sword and Behmer Labs, William Moar, and Heiko Vogel. This work was supported in part by the C. Everette Sayler Fellowship and the Charles Parencia Endowment through the Entomology Department at Texas A&M University.

CHAPTER VI

CONCLUSIONS

The four studies outlined in this dissertation culminate in three major conclusions concerning the effect of nutrition on insect stress response. First, insect herbivores feed in a nutritionally heterogeneous landscape that contains resources which constitute a range of optimality. Second, insect herbivores do not feed randomly in this nutritional landscape, and instead have evolved pre- and post-ingestive mechanisms to acquire the right balance of nutrients, particularly protein and carbohydrates, that maximizes fitness. Lastly, the ability to acquire a proper balance of macronutrients can have strong impacts on an insect's ability to deal with stress.

Chapter 2 showed that the nutritional environment in which insect herbivores forage in is highly variable in terms of plant macronutrients. Even in a cotton monoculture there exists significant variation in plant protein and carbohydrate content, not only spatially across tissues and environments, but also temporally throughout plant development. This means that an insect feeding on a cotton plant at any point in time has access to a range of macronutrient concentrations and ratios via different tissues or sub-tissues within specific structures, like the developing seeds found in bolls, that vary substantially, but also through access to tissues of different ages that vary over the height of the plant. This level of variability in plant macronutrients provides an opportunity for insect herbivores to mix diets and ingest the composite nutritional profiles that optimize their fitness.

In fact, the results of both Chapters 3 and 4 show that insects do indeed feed selectively. I looked at macronutrient regulation in two polyphagous cotton pest species. The first was *Lygus hespersus* (Western tarnished plant bug), which is a hemimetabolous sucking insect that uses pre-oral digesting and lacerating movements of its stylet to feed on plant cells. The second was *Helicovpera zea* (cotton bollworm), which is a homometabolous chewing insect. Despite having very different life histories, I found that both species actively regulated their intake of protein and carbohydrates, selecting for a specific balance, or intake target (IT). In both cases I found that they had slightly p-biased ITs, with *H. zea* selecting for a 1.6:1 p:c ratio and *L. hesperus* 1.2:1. This isn't particularly surprising given that both of these species tend to feed on p-rich fruiting structures.

In Chapter 5, the final chapter, I explored how diet macronutrients impact susceptibility to toxins, in this case the Cry1Ac endotoxin expressed in transgenic *Bt* cotton. Using *H. zea* as a model, results showed that macronutrient nutrition can have profound effects on how insects deal with stress. When Cry1Ac was present in lethal concentrations, *H. zea* larvae had the best survival on the diets that most closely matched their IT; however, as concentrations increased, the high macronutrient diet (which also matched the IT) showed significantly higher survival. Interestingly, under control conditions the high macronutrient diet produced significant wing deformations, suggesting that the best diets under stressful conditions may not be the best diets under non-stressful conditions.

Together, these studies indicate that macronutrient nutrition is an important

factor in insect stress response, and that opportunities exist for insects to regulate their macronutrient intake in order to mitigate the effects of stress. While I only explored the connection between macronutrients and stress for insects that could not diet mix, future work should focus on looking at feeding behavior, diet mixing, and macronutrient regulation in individuals that are under stress to determine the full impacts of nutrition. This research is incredibly important in agricultural systems where insecticide failures threaten our ability to provide food to an ever-increasing world population. While the primary concern in the agricultural sector currently is the development of insecticide resistance, these data show that nutritional plasticity may also have strong effects on pesticide efficacy. This is not only true because trait plasticity can contribute to genetic diversity and speciation, but also because variability in nutrition will likely also impact insect strains that harbor resistant alleles. Insecticide resistance management (IRM) relies heavily on diet-incorporation assays to test susceptibility of field populations to a variety of insecticides, including the Cry endotoxins found in *Bt* crops. Currently, little attention is given to the diets that are used in these studies or to their macronutrient composition. There is no standardized diet, and the majority of diets being used for lepidopteran assays are inexpensive rearing diets that are very c-biased and similar to the diets that we found produced the worst survival and performance in our study. These diets likely do not represent what larvae are feeding on in the field and populations may appear more susceptible than they really are due solely to the use of sub-optimal diets in these assays. This has important implications for IRM, as the use of improper diets can reduce our ability to detect resistance if it is present.

REFERENCES

Abisgold, J.D., Simpson, S.J., and Douglas, A.E. 1994. Nutrient regulation in the pea aphid *Acyrthosiphon pisum*: application of a novel geometric framework to sugar and amino acid consumption. Physiological Entomology, 19: 95-102.

Adamczyk Jr, J.J., Adams, L.C., and Hardee, D.D. 2001. Field efficacy and seasonal expression profiles for terminal leaves of single and double *Bacillus thuringiensis* toxin cotton genotypes. Journal of Economic Entomology, 94: 1589-1593.

Adang, M.J., Crickmore, N., Jurat-Fuentes, J.L. 2014. Diversity of *Bacillus thuringiensis* crystal toxins and mechanism of action. Advances in Insect Physiology, 47: 39-87.

Agusti, N. and Cohen, A.C. 2000. *Lygus hesperus* and *L. lineolaris* (Hemiptera: Miridae), phytophages, zoophages, or omnivores: evidence of feeding adaptations suggested by the salivary and midgut digestive enzymes. Journal of Entomological Science, 35: 176-186.

Ali, M.I., Luttrell, R.G. and Abel, C. 2007. Monitoring *Bt* susceptibilities in *Helicoverpa zea* and *Heliothis virescens*: results of 2006 studies, pp. 1062-1072. In Proceedings, 2007 Beltwide Cotton Conferences (9-12 January 2007, New Orleans, LA).

Ali, M.I., Luttrell, R.G., and Young III, S.Y. 2006. Susceptibilities of *Helicoverpa zea* and *Heliothis virescens* (Lepidoptera: Noctuidae) populations to Cry1Ac insecticidal proteins. Journal of Economic Entomology, 99: 164-175.

Altaye, S.Z., Pirk, C.W., Crewe, R.M., and Nicolson, S.W. 2010. Convergence of carbohydrate-biased intake targets in caged worker honeybees fed different protein sources. The Journal of Experimental Biology, 213: 3311-3318.

Anilkumar, K.J., Pusztai-Carey, M., and Moar, W.J. 2008. Fitness costs associated with Cry1Ac-resistant *Helicoverpa zea* (Lepidoptera: Noctuidae): a factor countering selection for resistance to *Bt* cotton? Journal of Economic Entomology, 101: 1421-1431.

Armstrong, J.S., and De Azevedo Camelo, L. 2003. *Lygus* species associated with Texas High Plains cotton, alfalfa and weeds. Southwestern Entomologist, 28: 197-204.

Auclair, J.L., and Raulston, J.R. 1966. Feeding of *Lygus hesperus* (Hemiptera: Miridae) on a chemically defined diet. Annals of the Entomological Society of America, 59: 1016-1017.

Backus, E.A., Cline, A.R., Ellerseick, M.R., and Serrano, M.S. 2007. *Lygus hesperus* (Hemiptera: Miridae) feeding on cotton: new methods and parameters for analysis of nonsequential electrical penetration graph data. Annals of the Entomological Society of America 100: 296-310.

Bazazi, S., Buhl, J., Hale, J. J., Anstey, M. L., Sword, G. A., Simpson, S. J., and Couzin, I. D. 2008. Collective motion and cannibalism in locust migratory bands. Current Biology, 18: 735-739.

Behmer, S.T. 2009. Insect Herbivore Nutrient Regulation. Annual Review of Entomology, 54: 167-187.

Behmer, S.T. and Joern, A. 2008. Coexisting generalist herbivores occupy unique nutritional feeding niches. Proceedings of the National Academy of Sciences of the United States of America, 105: 1977-1982.

Behmer, S.T., Raubenheimer, D. and Simpson, S.J. 2001. Frequency-dependent food selection in locusts: a geometric analysis of the role of nutrient balancing. Animal Behaviour, 61: 995-1005.

Behmer, S.T., Simpson, S.J., and Raubenheimer, D. 2002. Herbivore foraging in chemically heterogeneous environments: nutrients and secondary metabolites. Ecology, 83: 2489-2501.

Bell, A. 1986. Physiology of secondary products. Cotton Physiology. The Cotton Foundation, Memphis, TN, pp 597-621.

Benedict, J.H., Altman, D.W., Umbeck, P.F., and Ring, D.R. 1992. Behavior, growth, survival, and plant injury by *Heliothis virescens* (F.) (Lepidoptera: Noctuidae) on transgenic *Bt* cottons. Journal of Economic Entomology, 85: 589-593.

Benedict, J.H., Sachs, E.S., Altman, D.W., Ring, D.R., Stone, T.B., and Sims, S.R. 1993. Impact of σ-endotoxin-producing transgenic cotton on insect-plant interactions with *Heliothis virescens* and *Helicovpera zea* (Lepidoptera: Noctuidae). Environmental Entomology, 22: 1-9.

Berdegué, M. Trumble, J.T., and Moar, W.J. 1996. Effect of Cry1C toxin from *Bacillus thuringiensis* on larval feeding behavior of *Spodoptera exigua*. Entmologia Experimentalis et Applicata, 80: 389-401.

Berenbaum, M.R. 1983. Coumarins and caterpillars: a case for coevolution. Evolution, 37: 163-179.

Berenbaum, M.R. 1995. Turnabout is fair play: secondary roles for primary compounds. Journal of Chemical Ecology, 21: 925-940.

Bernays, E.A. and Bright, K.L. 1993. Mechanisms of dietary mixing in grasshoppers: a review. Comparative Biochemistry and Physiology, 104: 125-131.

Bernays, E.A. and Bright, K.L. 2005. Distinctive flavours improve foraging efficiency in the polyphagous grasshopper, *Taeniopoda eques*. Animal Behaviour, 69: 463–469.

Bernays, E.A. and Chapman, R.F. 1994. Host-Plant Selection by Phytophagous Insects. Chapman & Hall: New York, NY.

Bernays, E.A. and Minkenberg, O P.J.M. 1997. Insect herbivores: different reasons for being a generalist. Ecology, 78: 1157-1169.

Blanco, C.A., Gould, F., Vega-Aquino, P., Jurat-Fuentes, J.L., Perera, O.P., and Abel, C.A. 2009. Response of *Heliothis virescens* (Lepidotpera: Noctuidae) strains to *Bacillus thuringiensis* Cry1Ac incorporated into different insect artificial diets. Journal of Economic Entomology, 102: 1599-1606.

Boege, K. and Marquis, R.J. 2005. Facing herbivory as you grow up: the ontogeny of resistance in plants. Trends in Ecology & Evolution, 20: 441-448.

Boisen, S., Bech-Andersen, S., and Eggum, B.O. 1987. A critical view of the conversion factor 6.25 from total nitrogen to protein. Acta Agriculturae Scandinavica, 37: 299-304.

Bottrell, D.G. and Adkisson, P.L. 1977. Cotton insect pest management. Annual Review of Entomology, 22: 451-481.

Bradford, M.M. 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry, 72: 248-254.

Bradshaw, A.D. 1965. Evolutionary significance of phenotypic plasticity in plants. Advances in Genetics, 13: 115-155.

Burton, R.L. 1969. Mass rearing the corn earworm in the laboratory. U.S. Dep. Agriculture-ARS, 33: 134.

Celorio-Mancera, M.D.L.P., Carl Greve, L., Teuber, L.R., and Labavitch, J.M. 2009. Identification of endo-and exo-polygalacturonase activity in *Lygus hesperus* (Knight) salivary glands. Archives of Insect Biochemistry and Physiology, 70: 122-135.

Chevin, L., Lande, R., and Mace, G.M. 2010. Adaptation, plasticity, and extinction in a changing environment: towards a predictive theory. PLOS Biology, 8: e1000357.

Cline, A.R. and Backus, E.A. 2002. Correlations among AC electronic monitoring waveforms, body postures, and stylet penetration behaviors of *Lygus hesperus* (Hemiptera: Miridae). Environmental Entomology, 31: 538-549.

Cohen, A.C. 2000 (a). New oligidic production diet for *Lygus hesperus* Knight and L. lineolaris (Palisot de Beauvois). Journal of Entomological Science, 35: 301-310.

Cohen, A.C. 2000 (b). A review of feeding studies of *Lygus* spp. with emphasis on artificial diets. Southwestern Entomologist, 23: 111-119.

Compton, S.J. and Jones, C.G. 1985. Mechanism of dye response and interference in theBradford protein assay. Analytical Biochemistry, 151: 369-374.

Cotter, S.C., Simpson, S.J., Raubenheimer, D., and Wilson, K. 2010. Macronutrient balance mediates trade-offs between immune function and life history traits. Functional Ecology, 25:186-198.

Clissold, F.J., Sanson, G.D., and Read, J. 2006. The paradoxical effects of nutrient ratios and supply rates on an outbreaking insect herbivore, the Australian plague locust. Journal of Animal Ecology, 75(4): 1000-1013.

Clissold, F.J., Tedder, B.J., Conigrave, A.D., and Simpson, S.J. 2010. The gastrointestinal tract as a nutrient-balancing organ. Proceedings of the Royal Society B, 277: 1751-1759.

Cunha, F.M., Wanderly-Teixeira, V., Torres, J.B., Teixiera, Á.A., Alves, T.J., and Brayner, F.W. 2013. Impact of *Bt* cotton on the immune system and histology of the midgut of the fall armyworm *Spodoptera frugiperda* (JE Smith) (Lepidoptera: Noctuidae). Animal Biology, 63: 185-197.

Deans, C.A. 2014. The interaction between nutrition and stress response in insects (unpublished doctoral dissertation). Texas A&M University, Texas.

Deans, C.A., Behmer, S.T. and Sword, G.A. 2015. Revisiting macronutrient regulation in the polyphagous herbivore *Helicoverpa zea* (Lepidoptera: Noctuidae): new insights via a geometric approach. *submitted.*

Deans, C.A., Sword, G.A., Fiene, J. and Behmer, S.T. Spatio-temporal, genotypic and environmental effects on plant protein and digestible carbohydrate content: implications for insect herbivores. *in prep*.

Debolt, J.W. 1982. Meridic diet for rearing successive generations of *Lygus hesperus*. Annals of the Entomological Society of America, 75: 119-122.

Despland, E. and Noseworthy, M. 2006. How well do specialist feeders regulate nutrient intake? Evidence from a gregarious tree-feeding caterpillar. The Journal of Experimental Biology, 209: 1301-1309.

Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A., and Smith, F. 1956. Colormetric method for determination of sugars and related substances. Analytical Chemistry, 28: 350-358.

Eaton, F.M. and Ergle, D.R. 1953. Relationship of seasonal trends in carbohydrate and nitrogen levels and effects of girdling and spraying with sucrose and urea to the nutritional interpretation of boll shedding in cotton. Plant Physiology, 28: 503-520.

Ehrlich, P.R. and Raven, P.H. 1964. Butterflies and plants: a study in coevolution. Evolution, 18: 586-608.

Elser, J.J., Fagan, W.F., Denno, R.F., Dobberfuhl, D.R., Folarin, A., Huberty, A., Interlandi, S., Kilham, S.S., McCauley, E., Schulz, K.L., Siemann, and Sterner, R.W. 2000. Nutritional constraints in terrestrial and freshwater food webs. Nature, 408: 578-580.

Esquivel, J.F. 2015. Stylet penetration estimates for a suite of phytophagous hemipteran pests of row crops. Environmental Entomology, 27.

Esquivel, J.F. and Mowery, S.V. 2007. Host plants of the tarnished plant bug (Heteroptera: Miridae) in Central Texas. Environmental Entomology, 36: 725-730.

Ezeagu, I.E., Petzke, J.K., Metges, C.C., Akinsoyinu, A.O., and Ologhobo, A.D. 2002. Seed protein contents and nitrogen-to-protein conversion factors for some uncultizated tropical plant seeds. Food Chemistry, 78: 105-109.

Fitt, G.P. 1989. The ecology of *Heliothis* species in relation to agroecosystems. Annual Review of Entomology, 34: 17-52.

Fournier, A., Ellsworth, P.C., and Barkley, V.M. 2007. Economic impact of Lygus in Arizona cotton: a comparative approach. Cotton: A College of Agriculture and Life Sciences Report, 2007.

Fridgen, J.L. and Varco, J.J. 2004. Dependency of cotton leaf nitrogen, chlorophyll, and reflectance on nitrogen and potassium availability. Agronomy Journal, 96: 63-69.

Gibson, G. 2008. The environmental contribution to gene expression profiles. Nature Reviews, 9: 575-581.

Ghalambor, C.K., McKay, J.K., Carroll, S.P., and Reznick, D.N. 2007. Adaptive versus non-adaptive phenotypic plasticity and the potential for contemporary adaptation in new environments. Functional Ecology, 21: 394-407.

Gomulkiewicz, R. and Kirkpatrick, M. 1992. Quantitative genetics and the evolution of reaction norms. Evolution, 46: 390-411.

Gordon, H.T. 1961. Nutritional factors in insect resistance to chemicals. Annual Review of Entomology, 6: 27-54.

Gore, J., J. J. Adamczyk Jr, and C. A. Blanco. 2005. Selective feeding of tobacco budworm and bollworm (Lepidoptera: Noctuidae) on meridic diet with different concentrations of Bacillus thuringiensis proteins. Journal of Economic Entomology 98: 88-94.

Gore, J., Leonard, B.R., Church, G.E., and Cook, D.R. 2002. Behavior of bollworm (Lepidoptera: Noctuidae) larvae on genetically engineered cotton. Journal of Economic Entomology, 95: 763-769.

Gould, F., Anderson, A., Landis, D., and Mellaert, H.V. 1991. Feeding behavior and growth of *Heliothis virescens* larvae on diets containing *Bacillus thuringiensis* formulations or endotoxins. Entomologia Experimentalis et Applicata, 58: 199-210.

Greene, J.K., Turnipseed, S.G., Sullivan, M.J., and Herzog, G.A. 1999. Boll damage by Southern green stink bug (Hemiptera: Pentatomidae) and tarnished plant bug (Hemiptera: Miridae) cages on transgenic *Bacillus thuringiensis* cotton. Journal of Economic Entomology, 92: 941-944.

Greenplate, J.T. 1999. Quantification of *Bacillus thuringiensis* insect control protein Cry1Ac over time in Bollgard cotton fruit and terminals. Journal of Economic Entomology, 92: 1377-1383.

Güsewell, S. 2004. N:P ratios in terrestrial plants: variation and functional significance. New Phytologist, 164: 243-266.

Guttal, V., Romanczuk, P., Simpson, S. J., Sword, G. A., and Couzin, I. D. 2012. Cannibalism can drive the evolution of behavioural phase polyphenism in locusts. Ecology Letters, 15: 1158-1166.

Hägele, B.F. and Rowell-Rahier, M. 1999. Dietary mixing in three generalist herbivores: nutrient complementation or toxin dilution. Oecologia, 119: 521-533.

Halevy, J., Marani, A., and Markovitz, T. 1987. Growth and NPK uptake of high-yielding cotton grown at different nitrogen levels in a permanent-plot experiment. Plant and Soil, 103: 39-44.

Hansen, M. J., Buhl, J., Bazazi, S., Simpson, S. J., & Sword, G. A. 2011. Cannibalism in the lifeboat—collective movement in Australian plague locusts. Behavioral Ecology and Sociobiology, 65: 1715-1720.

Hewitt, E. and Smith, T.A. 1974. Plant mineral nutrition. English Universities Press Ltd., London, UK.

Izhaki, I. 1993. Influence of nonprotein nitrogen on estimation of protein from total nitrogen in fleshy fruits. Journal of Chemical Ecology, 19: 2605-2615.

Jermy, T. 1984. Evolution of insect/host plant relationships. The American Naturalist, 124: 609.

Jing, X., Grebenok, R.J., and Behmer, S.T. 2013. Sterol/steroid metabolism and absorption in a generalist and specialist caterpillar: effects of dietary sterol/steroid structure, mixture, and ratio. Insect Biochemistry and Molecular Biology, 43: 580-587.

Jing, X., Vogel, H., Grebenok, R.J., Zhu-Salzman, K., and Behmer, S.T. 2012. Dietary sterols/steroids and the generalist caterpillar *Helicoverpa zea*: physiology, biochemistry and midgut gene expression. Insect Biochemistry and Molecular Biology, 42: 835-845.

Joham, H.E. 1951. The nutritional status of the cotton plant as indicated by tissue tests. Plant Physiology, 26: 76-89.

Jones, S.A., Raubenheimer, D. 2001. Nutritional regulation in nymphs of the German cockroach, *Blattella germanica*. Journal of Insect Physiology, 47: 1169-1180.

Karban, R. and Baldwin, I.T. 2007. Induced Responses to Herbivory. University of Chicago Press: Chicago, IL, USA.

King, E.G, Hartley, G.G., Martin, D.F. and Laster, M.L. 1985. Large-scale rearing of a sterile backcross of the tobacco budworm (Lepidoptera: Noctuidae). Journal of Economic Entomology, 78: 1166-1172.

Kotkar, H.M., Sarate, P.J., Tamhane, V.A., Gupta, V.S., and Giri, A.P. 2009. Responses of midgut amylases of *Helicoverpa armigera* to feeding on various host plants. Journal of Insect Physiology, 55: 663-670.

Landes, D.A. and Strong, F.E. 1965. Feeding and nutrition of *Lygus hesperus* (Hemiptera: Miridae). I. Survival of bugs fed on artificial diets. Annals of the Entomological Society of America, 58: 306-309.

Layton, M.D. 2000. Biology and damage of the tarnished plant bug, *Lygus lineolaris*, in cotton. Southwestern Entomologist, 23: 7-20.

Layton, M.B., Long, J.L., and Green, L.M. 2003. Control of tarnished plant bugs on cotton, 2002. Arthropod Management Tests, 28: F66.

Leclaire, M. and Brandle, R. 1994. Phenotypic plasticity and nutrition in a phytophagous insect: consequences of colonizing a new host. Oecologia, 100: 379-385.

Lee, K.P. 2007. The interactive effects of protein quality and macronutrient imbalance on nutrient balancing in an insect herbivore. Journal of Experimental Biology, 219: 3236–3244.

Lee, K.P. 2010. Sex-specific differences in nutrient regulation in a capital breeding caterpillar, *Spodoptera litura* (Fabricius). Journal of Insect Physiology, 56: 1685–1695.

Lee, K.P., Behmer, S.T., and Simpson, S.J. 2006. Nutrient regulation in relation to diet breadth: a comparison of *Heliothis* sister species and a hybrid. The Journal of Experimental Biology, 209: 2076-2084.

Lee, K.P., Behmer, S.T., Simpson, S.J., and Raubenheimer, D. 2002. A geometric analysis of nutrient regulation in the generalist caterpillar *Spodoptera littoralis* (Boisduval). Journal of Insect Physiology, 48: 655-665.

Lee, K.P., Cory, J.S., Wilson, K., Raubenheimer, D., and Simpson, S.J. 2006. Flexible diet choice offsets protein costs of pathogen resistance in a caterpillar. Proceedings of the Royal Society B, 273:823-829.

Lee, K.P., Kwon, S.T. & Roh, C. 2012. Caterpillars use developmental plasticity and diet choice to overcome the early life experience of nutritional imbalance. Animal Behaviour, 84: 785–793.

Lee, K.P., Raubenheimer, D., Behmer, S.T. & Simpson, S.J. 2003. A correlation between macronutrient balancing and insect host-plant range: evidence from the specialist caterpillar *Spodoptera exempta* (Walker). Journal of Insect Physiology, 49: 1161–1171.

Lee, K.P., Raubenheimer, D., and Simpson, S.J. 2004. The effects of nutritional imbalance on compensatory feeding for cellulose-mediated dietary dilution in a generalist caterpillar. Physiological Entomology, 29: 108-117.

Lee K.P., Simpson S.J., Raubenheimer D. 2004. A comparison of nutrient regulation between solitarious and gregarious phases of the specialist caterpillar, *Spodoptera exempta* (Walker). Journal of Insect Physiology, 50: 1171–1180.

Lee, K.P., Simpson, S.J., and Wilson, K. 2008. Dietary protein-quality influences melanization and immune function in an insect. Functional Ecology, 22: 1052-1061.

Le Gall, M. and Behmer, S.T. 2014. Effects of protein and carbohydrate on an insect herbivore: the vista from a fitness landscape. Integrative and Comparative Biology, 54: 942-954..

Leigh, T.F., Kerby, T.A., and Wynholds, P.F. 1988. Cotton square damage by the plant bug, *Lygus hesperus* (Hemiptera: Heteroptera: Miridae), and abscission rates. Journal of Economic Entomology, 81: 1328-1337.

Levins, R. 1968. Evolution in Changing Environments: Some Theoretical Explorations (No. 2). Princeton University Press: Princeton, NJ.

Li, Y.X., Greenberg, S.M. and Liu, T.X. 2006. Effects of *Bt* cotton expressing Cry1Ac and Cry2Ab and non-*Bt* cotton on behavior, survival and development of *Trichoplusia ni* (Lepidoptera: Noctuidae). Crop Protection, 25: 940-948.

Li. R., Volenec, J.J., Joern, B.C., and Cunningham, S.M. 1996. Seasonal changes in nonstructural carbohydrates, protein, and macronutrients in roots of alfalfa, red clover, sweetclover, and birdsfoot trefoil. Crop Science, 36: 617-623.

Lihoreau, M., Buhl, J., Charleston, M. A., Sword, G. A., Raubenheimer, D., and Simpson, S. J. 2014. Modelling nutrition across organizational levels: From individuals to superorganisms. Journal of Insect Physiology, 69: 2-11.

Lihoreau, M., Buhl, J., Charleston, M. A., Sword, G. A., Raubenheimer, D., and Simpson, S. J. 2015. Nutritional ecology beyond the individual: a conceptual framework for integrating nutrition and social interactions. Ecology Letters, 18: 273-286.

Lively, C.M. 1986. Canalization versus developmental conversion in a spatially variable environment. American Naturalist, 128: 561-572.

Luttrell, R. G. and M. I. Ali. 2007. Exploring selection for *Bt* resistance in heliothines: results of laboratory and field studies, pp. 1073-1086. In Proceedings, 2007 Beltwide Cotton Conferences (9-12 January 2007, New Orleans, LA).

Luttrell, R.G. and Jackson, R.E. 2012. *Helicoverpa zea* and Bt cotton in the United States. GM Crops Food, 3: 213-227.

Luttrell, R. G., L. Wan and K. Knighten. 1999. Variation in susceptibility of noctuid (Lepidoptera) larvae attacking cotton and soybean to purified endotoxin proteins and commercial formulations of *Bacillus thuringiensis*. Journal of Economic Entomology, 92: 21- 32.

Matthews, G.A. 1989. Cotton insect pests and their management. Longman Scientific and Technical: Harlow, UK.

McGroddy, M.E., Daufresne, T., and Hedin, L.O. 2003. Scaling of C:N:P stoichiometry in forests worldwide: implications of Redfield-type ratios. Ecology, 85: 2390-2401.

Merkx-Jacques, M., Despland, E., and Bede, J. 2008. Nutrient utilization by caterpillars of the generalist beet armyworm, *Spodoptera exigua*. Physiological Entomology, 33: 51-61.

Moar, W., Roush, R., Shelton, A., Ferré, J., MacIntosh, S., Leonard, B.R. and Abel, C. 2008. Field-evolved resistance to *Bt* toxins. Nature Biotechnology, 26: 1072-1074.

Moran, N.A. 1992. The evolutionary maintenance of alternative phenotypes. American Naturalist, 139: 971-989.

Mossé, J. 1990. Nitrogen to protein conversion factor for ten cereals and six legume or oilseeds. A reappraisal of its definition and determination. Variation according to species and to seed protein content. Journal of Agricultural Food Chemistry, 38: 18-24.

Mullins, G.L. and Burmester, C.H. 1990. Dry matter, nitrogen, phosphorus, and potassium accumulation by four cotton varieties. Agronomy Journal, 82: 729-736.

Orpet, R.J., Degain, B.A., Unnithan, G.C., Welch, K.L., Tabashnik, B.E., and Carrière, Y. 2015. Effects of dietary protein to carbohydrate ratio on *Bt* toxicity and fitness costs of resistance in *Helicoverpa zea*. Entomologia Experimentalis et Applicata, *in press*.

Parker, C.D. and Luttrell, R.G. 1999. Interplant movement of *Heliothis virescens* (Lepidoptera: Noctuidae) larvae in pure and mixed plantings of cotton with and without expression of Cry1Ac σ-endotoxin protein of *Bacillus thuringiensis* Berliner. Journal of Economic Entomology, 92: 837-845.

Pigliucci, M. 2001. Pheotypic Plasticity: Beyond Nature and Nurture. The John Hopkins University Press: London, UK.

Ponton, F., Wilson, K., Cotter, S.C., Raubenheimer, D., and Simpson, S.J. 2011. Nutritional immunology: a multi-dimensional approach. PLoS Pathogens, 7: e1002223.

Ponton, F., Wilson, K., Holmes, A.J., Cotter, S.C., Raubenheimer, D., and Simpson, S.J. 2013. Integrating nutrition and immunology: a new frontier. Journal of Insect Physiology, 59: 130-137.

Povey, S., Cotter, S.C., Simpson, S.J., Lee, K.P., and Wilson, K. 2008. Can the protein costs of bacterial resistance be offset by altered feeding behavior? Journal of Animal Ecology, 78:437-446.

Price, T.D., Qvarnström, A., and Irwin, D. 2003. The role of phenotypic plasticity in driving genetic evolution. Proceedings of the Royal Society of London. Series B: Biological Sciences, 270: 1433-1440.

Raubenheimer, D. 1992. Tannic acid, protein, and digestible carbohydrate: dietary imbalance and nutritional compensation in locusts. Ecology, 73: 1012-1027.

Raubenheimer, D. and Jones, S.A. 2006. Nutritional imbalance in an extreme generalist omnivore: tolerance and recovery through complementary food selection. Animal Behaviour, 71: 1253-1262.

Raubenheimer, D. and Simpson S.J. 1999. Integrating nutrition: a geometrical approach. Entomologica Experimentalis Applicata, 91: 67–82

Raubenheimer, D. and Simpson, S.J. 2003. Nutrient balancing in grasshoppers: behavioural and physiological correlates of dietary breadth. The Journal of Experimental Biology, 206: 1669-1681.

Raulston, J.R., and Auclair, J.L. 1968. Responses of *Lygus hesperus* to chemically defined diets. Annals of the Entomological Society of America, 61: 1495-1500.

Ritter, K.S. and Nes, W.R. 1981. The effects of the structure of sterols on the development of *Heliothis zea*. Journal of Insect Physiology, 27: 419-424.

Roeder, K.A. and Behmer, S.T. 2014. Lifetime consequences of food protein-carbohydrate content for an insect herbivore. Functional Ecology, 28: 1135-1143.

Rosenheim, J.A., Steinmann, K., Langellotto, G.A., and Zink, A.G. 2006. Estimating the impact of *Lygus hesperus* on cotton: the insect, plant, and human observer as sources of variability. Environmental Entomology, 35: 1141-1153.

Sánchez, E., Rivero, R.M., Ruiz, J.M., and Romero, L. 2004. Changes in biomass, enzymatic activity and protein concentration in roots and leaves of green bean plants (*Phaseolus vulgaris* L. cv. Strike) under high NH4NO3 application rates. Scientia Horticulturae, 99: 237-248.

Sarate, P.J., Tamhane, V.A., Kotkar, H.M., Ratnakaran, N., Susan, N., Gupta, V.S., and Giri, A.P. 2012. Developmental and digestive flexibilities in the midgut of a polyphagous pest, the cotton bollworm, *Helicoverpa armigera*. Journal of Insect Science, 12: 1-16.

Sayyed, A. H., Cerda, H., and Wright, D.J. 2003. Could *Bt* transgenic crops have nutritionally favourable effects on resistant insects? Ecology Letters, 6: 167-169.

Schlichting, C.D. and Pigliucci, M. 1998. Phenotypic evolution: a reaction norm perpective. Sinauer Associates Inc., Sunderland, MA.

Schoonhoven, L.M. 1968. Chemosensory bases of host plant selection. Annual Review of Entomology, 13: 115-136.

Schoonhoven, L.M., van Loon, J.J.A. and Dicke, M. 2005. Insect-Plant Biology. Oxford University Press.

Shikano, I. and Cory, J.S. 2013. Genetic resistance to *Bacillus thuringiensis* alters feeding behavior to the cabbage looper, *Trichoplusia ni*. PLOS One, 9: e85709.

Shikano, I. and Cory, J.S. 2014. Dietary mechanisms behind the costs associated with resistance to *Bacillus thuringiensis* in the cabbage looper, *Trichoplusia ni*. PLOS One, 9: e105864.

Simpson, S.J. and Raubenheimer, D. 1993. The central role of the haemolymph in the regulation of nutrient intake in insects. Physiological Entomology, 18: 395-403.

Simpson, S.J. and Raubenheimer, D. 1995. The geometric analysis of feeding and nutrition: a user's guide. Journal of Insect Physiology, 41: 545-553.

Simpson, S.J. and Raubenheimer, D. 1999. Assuaging nutritional complexity: a geometrical approach. Proceedings of the Nutrition Society, 58:779–89.

Simpson, S.J. and Raubenheimer, D. 2001. The geometric analysis of nutrient-allelochemical interactions: a case study using locusts. Ecology, 82: 422-439.

Simpson, S.J. and Raubenheimer, D. 2009. Nutrition, ecology and nutritional ecology: towards an integrated framework. Functional Ecology, 23: 4-16.

Simpson, S.J., Raubenheimer, D., Behmer, S.T., Whitworth, A., and Wright, G.A. 2002. A comparison of nutritional regulation in solitarious- and gregarious-phase

nymphs of the desert locust *Schistocerca gregaria*. The Journal of Experimental Biology, 205: 121-129.

Simpson, S.J., Raubenheimer, D., Charleston, M.A, and Clissold, F.J. 2010. Modelling nutritional interactions: from individuals to communities. Trends in Ecology and Evolution, 25: 53-60.

Simpson, S.J., Sibly, R.M., Lee K.P., Behmer, S.T., Raubenheimer, D. 2004. Optimal foraging when regulating intake of multiple nutrients. Animal Behaviour, 68: 1299-1311.

Simpson, S.J., Sword, G.A., Lorch, P.D., and Couzin, I.D. 2006. Cannibal crickets on a forced march for protein and salt. . Proceedings of the National Academy of Sciences of the United States of America, 103: 4152-4156.

Snodgrass, G.L., Scott, W.P., and Smith, J.W. 1984. Host plants and seasonal distribution of the tarnished plant bug (Hemiptera: Miridae) in the Delta of Arkansas, Louisiana, and Mississippi. Environmental entomology, 13: 110-116.

Snodgrass, G.L. 1996. Insecticide resistance in field populations of the tarnished plant bug (Heteroptera:Miridae) in cotton in the Mississippi Delta. Journal of Economic Entomology, 89: 783-790.

Snodgrass, G.L. and Scott, W.P. 2000. Seasonal changes in pyrethroid resistance in tarnished plant bug (Heteroptera: Miridae) populations during a three-year period in the delta area of Arkansas, Louisiana, and Mississippi. Journal of Economic Entomology, 93: 441-446.

South, S.H., House, C.M., Moore, A.J., Simpson, S.J., and Hunt, J. 2011. Male cockroaches prefer a high carbohydrate diet that makes them more attractive to females: implications for the study of condition dependence. Evolution, 65: 1594-1606.

Stieger, P.A. and Feller, U. 1994. Senescence and protein remobilization in leaves of maturing wheat plants grown on waterlogged soil. Plant and Soil, 166: 173-179.

Strong, F.E., and Kruitwagen, E. 1969. Feeding and nutrition of *Lygus hesperus*. III. Limited growth and development on a meridic diet. Annals of the Entomological Society of America, 62: 148-155.

Strong, F.E. 1970. Physiology of injury caused by *Lygus hesperus*. Journal of Economic Entomology, 63: 808-814.

Strong, F.E. and Kruitwagen, E. 1968. Gustatory discrimination between meridic diets by the bug, *Lygus hesperus*. Journal of Insect Physiology, 16: 521-530.

Srygley, R. B., Lorch, P. D., Simpson, S. J., and Sword, G. A. 2009. Immediate protein dietary effects on movement and the generalised immunocompetence of migrating Mormon crickets *Anabrus simplex* (Orthoptera: Tettigoniidae). Ecological Entomology, 34: 663-668.

Tabashnik, B.E. 1994. Evolution of resistance to *Bacillus thuringiensis*. Annual Review of Entomology, 39: 47-79.

Tabashnik, B.E., Brévault, and Carrière, Y. 2013. Insect resistance to Bt crops: lessons from the first billion acres. Nature Biotechnology, 31: 510-521.

Tabashnik, B.E., Huang, F., Ghimire, M.N., Leonard, B.R., Siegfried, B.D., Rangasamy, M., Yang, Y., Wu, Y., Gahan, L.J., Heckel, D.G., Bravo, A., and

Soberón, M. 2011. Efficacy of genetically modified *Bt* toxins against insects with different genetic mechanisms of resistance. Nature Biotechnology, 29: 1128-1131.

Thompson, S.N. and Redak, R.A. 2005. Feeding behavior and nutrient selection in an insect *Manduca sexta* L. and alterations induced by parasitism. Journal of Comparative Physiology, 191: 909-923.

Travis, J. 1994. Evaluating the adaptive role of morphological plasticity. pp 99-122. In Ecological morphology: integrative organismal biology. University of Chicago Press: Chicago, IL.

Unsicker, S.B., Oswald, A., Köhler, G., and Weisser, W.W. 2008. Complementarity effects through dietary mixing enhance the performance of a generalist insect herbivore. Oecologia, 156: 313-324.

Vanderzant, E.S. 1967. Rearing *Lygus* bugs on artificial diets 1, 2. Journal of Economic Entomology, 60: 813-816.

Via, S. and Lande, R. 1985. Genotype-environment interaction and the evolution of phenotypic plasticity. Evolution, 39: 505-522.

Waldbauer, G.P., Cohen, R.W., and Friedman, S. 1984. Self-selection of an optimal nutrient mix from defined diets by larvae of the corn earworm. Physiological Zoology, 57: 590-597.

West-Eberhard, M.J. 2003. Developmental plasticity and evolution. Oxford University Press.

Wilson, L.T., Leigh, T.F., Gonzalez, D., Foristiere, C. 1984. Distribution of *Lygus hesperus* (Knight) (Miridae: Hemiptera) on cotton. Journal of Economic Entomology, 77: 1313-1319.

Xu, X., Yu, L., and Wu, Y. 2005. Disruption of cadherin gene associated with resistance to Cry1Ac σ-endotoxin of *Bacillus thuringiensis* in *Helicoverpa armigera*. Applied and Environmental Microbiology, 71: 948-954.

Young, O.P. 1986. Host plants of the tarnished plant bug, *Lygus lineolaris* (Heteroptera:Miridae). Annals of the Entomological Society of America, 79: 747-762.

Zhang, J.H., Wang, C.Z., Qin, J.D., and Guo, S.D. 2004. Feeding behavior of *Helicoverpa armigera* larvae on insect-resistant transgenic cotton and non-transgenic cotton. Journal of Applied Entomology, 128: 218-225.

Zink, A.G. and Rosenheim, J.A. 2005. Stage-dependent feeding behavior by western tarnished plant bugs influences flower bud abscission in cotton. Entomologia Experimentalis et Applicata, 117: 235-242.